Manual of Hemorrhagic Fever with Renal Syndrome

WHO Collaborating Center for Virus Reference and Research (Hemorrhagic fever with renal syndrome) Institute for Viral Diseases, Korea University.

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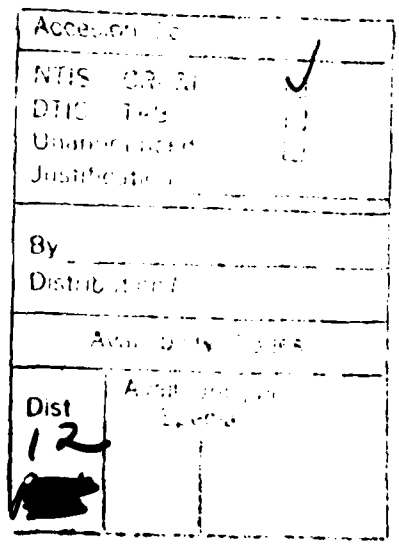

WHO Collaborating Center for Virus Reference and Research (Hemorrhagic fever with renal syndrome) Institute for Viral Diseases, Korea University.

Preface

The discovery of Hantaan virus in 1976 launched a new era in the study of hemorrhagic fever with renal syndrome (HFRS) throughout the world. Accumulated data indicate that there are about 200,000 HFRS patients in the world each year with a 3.7% case fatality.

We now know that hantaviruses are ubiquitous throughout the world and field mice, urban rats, and laboratory rats are reservoir hosts of HFRS. Although HFRS is still not recognized in certain parts of the world, it may be that many of these areas have either not intensively searched for hantaviruses or currently lack diagnostic capability.

This manual is the result of the efforts of numerous contributors. They have graciously undertaken the tedious and often time consuming task of preparing an entirely new manual. All of the contributors were selected because of their experience in this field and eminence as scientists as well as their special ability to describe their specific area of expertise in each particular section. The editors have not attempted to influence the author's choice of material considered or the information presented and have carefully avoided influencing either the view or the conclusion of the authors.

This manual is designed to provide comprehensive, basic information on the standard techniques for both research and diagnosis of HFRS. Hopefully, it meets the needs of both researchers and physicians in the field. It is further hoped that this manual will serve as the basis for a better understanding of HFRS and a stimuius for further research which must, in the end, lead us to mechanisms for prevention and control of this fatal human disease.

The editors are deeply grateful to all of the contributors for the enormous effort they expended and the care and thought they gave to the preparation of manuscripts. We are also grateful for the generous spirit of wholehearted cooperation which endured throughout the lengthy process of the development of this manual.

The editors wish to express their eternal gratitude to the World Health Organization, Western Pacific Region, for their support of the WHO Collaborative Centre for HFRS that has made all of this possible.

April 1989

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Foreword

Haemorrhagic Fever with Renal Syndrome (HFRS) is endemic in China, the Republic of Korea and other parts of the world in Europe and Asia, and is a threat to public health. The discovery of the etiological agent of HFRS, the Hantaan virus, was made by Dr Ho Wang Lee, Professor, Medical College, Korea University, Seoul, Republic of Korea, in 1976. It opened the way for the development of laboratory diagnostic methods for the detection of the agent and its activities, the epidemiological surveillance of HFRS infection and disease on a worldwide basis, and the development of control measures including the search for vaccines.

This manual on Haemorrhagic Fever with Renal Syndrome is the result of the joint efforts of several experts under the leadership of Dr Lee. It provides information on the history, distribution, virology, epidemiology, clinical diagnosis, management and laboratory diagnosis of Hantaan virus infection.

This infection appears to be more widely distributed than originally thought. The conduct of sero-surveys and the safety precautions needed in working with the virus are well described in these pages. They provide a very useful guide and reference point for virologists, epidemiologists, researchers, teachers and other health workers engaged in or studying the prevention and control of HFRS.

WHO expresses its thanks and appreciation to all those who have contributed to the preparation of this manual. In particular, we wish to congratulate Dr Ho Wang Lee for his efforts in producing the manual and for job that is indeed well done.

April 1989

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Manual of Hemorrhagic Fever with Renal Syndrome

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Chapter I

Introduction

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Introduction

1. Background

The isolation by Lee and Lee (1, 2) of the virus causing Korean hemorrhagic fever (KHF), now called Hantaan virus (3, 4), from the lungs of striped field mice (*Apodemus agrarius*), the wild rodent reservoir, launched a new era in the study of hemorrhagic fever with renal syndrome (HFRS) throughout the world. Studies have confirmed that the epidemic hemorrhagic fever in the people's Republic of China and the Far Eastern hemorrhagic nephroso-nephritis in the Soviet Union are caused by the same virus (4-7) and that HFRS in European Russia and the Balkan countries and nephropathia epidemica in Scandinavia are caused by Puumala virus, an antigenically related Bunyavirus that is distinguishable serologically from Hantaan virus (8-10). The nephropathia epidemica antigen has been detected (11) and the virus has been isolated from the lungs of the reservoir bank voles (*Clethrionomys glareolus*) (12, 13). In addition, serologic surveys (10) of patients in Sweden, Finland, Hungary, Yugoslavia, and European Russia have shown that both the Hantaan and Puumala serotypes are circulating in these areas.

The availability of Hantaan virus antigen has further permitted the diagnosis of urban cases throughout Korea, China, and Japan of a disease, transmitted to man from urban commensal rats (*Rattus norvegicus* and *Rattus rattus*), which is characterized by mild nephropathy with minimal shock or hemorrhagic diathesis or by only flu-like symptoms with albuminuria (14-18). Occasionally, however, some urban cases are severe (14, 16). This urban rat virus, now named Seoul virus, has been serologically identified in Africa, North and South America, India, the highlands of New Guinea,

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several Pacific islands (including Hawaii, Fiji, Philippines, and Taiwan), and major port cities of the continental United States. Isolation attempts have yieled Hantaan-like viruses from rats captured in the Americas (19-22) ; and although clinical disease has not been identified, serologic evidence of infection exists (20, 23). Surprisingly, almost 1% of healthy blood-bank donors in the United States have low titered fluorescent antibodies against Hantaan virus (23). In Japan, Belgium, France, and Korea, laboratory rats have been found to be chronically infected with the virus ; and in Japan, Belgium, Korea and the United Kingdom, clinical disease with an occasional fatality has been reported among investigators working with laboratory rats (24-28).

Thus, Hantaan and related viruses cause an acute viral nephropathy across much of the Eurasian landmass in the form of a hemorrhagic disease of great clinical severity. Mortality rates range from 5% to more than 20% in East Asia. Lower mortality rates are fonnd in nephropathia epidemica, a much milder form of non-hemorrhagic nephropathy in Scandinavia (29). Both mild and severe forms of HFRS occur in the Balkan countries and European Russia. Serologic surveys also reveal that infection with these viruses in humans may be clinically inapparent or atypical (30, 31).

A fourth serologic type, in addition to the Hantaan, Seoul, and Puumala virus, has now been isolated from meadow voles (*Microtus pennsylvanicus*) in the United States, and is called Prospect Hill virus (32). Serological evidence of Prospect Hill virus infection has been found in American mammalogists (33), but the clinical features of infection are unknown.

2. Terminology of virus hemorrhagic fevers

The viral hemorrhagic fevers are characterized by bleeding in a significant proportion of patients. They are caused by a diverse array of RNA-containing viruses belonging to the flavivirus, arenavirus, and bunyavirus families. Yellow fever, caused by a mosquito-borne flavivirus, although described

over a century ago, clearly belongs in the group of viral hemorrhagic fevers.

The disease that brought this designation and classification to the attention of American physicians was epidemic hemorrhagic fever, now called Korean hemorrhagic fever, and it is this and other diseases caused by viruses belonging to the newly defined Hantavirus genus of the family Bunyaviridae that are now called hemorrhagic fever with renal syndrome (HFRS). Since urban cases of hantavirus infections, acquired from contact with commensal rats in Korea, Japan, and China, and nephropathia epidemica of Scandinavia seldom show any bleeding phenomenon, the term hemorrhagic fever for these clinical entities is misleading. However, all known diseases caused by these viruses are characterized by proteinuria and azotemia. The virus is transmitted to humans by exposure to aerosols of infectious secretions and excretions from silently infected carrier rodents who shed the virus in their lungs, saliva, and urine (34). All these reservoir rodents belong to the superfamily *Muroidea* and the families *Muridae* (genera : *Apodemus* and *Rattus*) and *Cricetidae* (genera : *Clethrionomys* and *Microtus*). Thus, the name muroid virus nephropathies has also been suggested for this group of Hantavirus caused diseases (35).

3. History of HFRS

In 1932, the Russians observed a new disease along the lower Amur River basin in the southeastern part of the Soviet Far East. Thereafter, yearly outbreaks of the illness occurred in the Amur valley, which forms the boundary between Manchuria and the Soviet Union, and in the valleys of its tributaries (36). Shortly thereafter, across the Amur, along its Manchurian tributaries, the Japanese army encountered 12,000 cases of this disease among its one million troops. Research teams from both countries, studying the disease independently and without knowledge of each other's work, established its infectious, probable viral etiology and

defined its clinical and epidemiologic features (36 38). Both Russian and Japanese workers succeeded in producing typical disease in human volunteers by intravenous and intramuscular injection of blood and urine obtained from patients early in their illness (37, 39). Serial passage in humans was also successful, and experimental infection conferred immunity to subsequent challenge with the agent. They could not, however, establish the disease in experimental animals. A Russian expeditionary team studied the disease in the Soviet Far East in 1939 and 1940, while the Japanese studies dated from 1936 to 1942. The Russians called the disease by various names, including Churilov's disease, Far Eastern nephroso-nephritis and epidemic hemorrhagic nephroso nephritis. The Japanese, before settling on epidemic hemorrhagic fever (*ryukosei shukketsu netsu*), used a number of names based on the geographic locations of their cases : Ertaokiang disease (Nidoko fever), Songo (Sunwu) fever, and Tayinshan disease. American medical literature quickly adopted the term epidemic hemorrhagic fever, whereas the Russians tended to use the term hemorrhagic fever with renal syndrome in later years, instead of their earlier designation epidemic (Far Eastern) hemorrhagic nephrosonephritis.

In the spring of 1951, United Nations troops near the fighting front in Korea began to appear on sick call with chills and high fever, prostration, anorexia, severe retroorbital headache, generalized muscle and joint pains, vomiting, facial flushing, and diverse hemorrhagic manifestations, including petechiae, ecchymoses and scleral hemorrhages. Laboratory examination often revealed leukocytosis and marked proteinuria. The unusual and dramatic course of this severe, often fatal illness, with cardiovascular instability, shock, and renal failure, soon convinced physicians, who had first suspected leptospirosis, allergic and idiopathic thrombocytopenic purpura, leukemia, infectious mononucleosis, hemorrhagic smallpox, lower nephron nephrosis, and other renal disorders, that they were dealing with an infection hitherto unknown to Western medicine (35, 40).

By the end of 1951, this illness had taken 80 lives of the 1,000 men who had contracted it. A year later, another thousand cases were confirmed in Korea. All nationalities of the United Nations troops, including South Koreans, were affected. Cases continued to occur in 1953 and 1954 after hostilities had ceased : 3,200 cases involved United Nations forces. The disease continued to occur in troops and in the civilian population in subsequent years and soon became the major infectious disease problem of the Korean army, which took over surveillance of the Demilitarized Zc It occurred more frequently in the civilian population ana in locations further south in Korea than the area of endemicity recognized during the war. Although the initial mortality rate was over 10%, it dropped to about 5% with improved methods of intensive medical care, resulting from better the understanding of the disease and with recognition of milder forms of the illness (41).

During the Korean War, the American Army Medical Service established a Hemorrhagic Fever Center near Uijonbu, close to the region where the largest number of cases occurred in South Korea. All patients with suspected disease were evacuated by helicopter to the Center's hospital, where a careful, investigative program, which included treatment and other research, was conducted (42). American efforts, more extensive than the earlier attempts of the Russians and Japanese, likewise failed to establish the disease in any of a wide range of experimental hosts, including chimpanzees and monkeys, and continuous and primary animal and human cell cultures.

During the resettlement of the former combat zone, which had been evacuated during hostilities, cases of the disease were first observed in the civilian Korean populace. A few cases, mild in nature and short in duration, were even seen in children younger than 10 years of age. No evidence was found to suggest that a part of the population had acquired resistance to the disease through prior residence in the area in which it was known to occur.

In 1958 and 1959, Soviet physicians established that the clinical, pathologic, and epidemiologic features of the hemorrhagic fever, which they had been studying in the Yaroslav Oblast along the Upper Volga River and in the Urals of European Russia, were actually those of HFRS. They also recognized that it was the same disease they had previously called Tula fever : 915 cases of this disease, with 5 deaths, had been reported from 1930 to 1934 in and around Tula. Thus, Tula fever is the oldest described HFRS outbreak in the Soviet Union. The demonstration that HFRS was occurring over widely separated areas in eastern Europe, eastward to the Urals, northwestward to the Murmansk Oblast, and southwestward to Hungary, Czechoslovakia, Bulgaria, Romania, and Yugoslavia, strengthened the suspicion that nephropathia epidemica of Scandinavia was the same disease (43). Furthermore, the likelihood appeared great that between the Far Eastern foci in the Soviet Far East, northern China (Manchuria), and Korea and the foci in Europe, further foci would be found. Chiu and colleagues (44) in 1957 reported cases of HFRS in Inner Mongolia in the Peoples's Republic of China. The revelations that, since 1963, the disease has seen as a major problem in southern and central China and that, by 1981, it was recognized in 19 provinces of China confirmed earlier predictions that the disease would be found widespread in Europe and Asia.

4. Current status of virus classification

Sequencing of the 3' terminal nucleotides of the three segments of single-stranded RNA of the four known major serotypes has shown that they share common sequences and form a new genus (*Hantavirus*) of the Bunyaviridae family, with close sequence similarity to the type virus Bunyamwera (45). These four serotypes are each adapted to the Vero E6 cell line, which has permitted the typing of new isolates by the immunofluorescent antibody technique, plaque reduction neutralization test, radioimmunoassay, enzyme linked

immunosorbent assay, hemadsorption and hemagglutination inhibition (HI) test.

The close antigenic relationship of the four serotypes is evident from cross fluorescent antibody tests (45, 46), although it is often a "one-way cross". In contrast, they are less closely related or do not cross at all when blocking antibody and neutralization tests are used (45, 46).

Recent work using monoclonal antibodies indicates strain differences among most isolates of Hantaviruses. Thus, monoclonal antibodies provide a tool for distinguishing virus strains and isolates, while blocking antibodies and neutralizing antibody tests provide a means of discriminating among serotypes (32, 46). The IFA technique remains the single most reliable assay for detecting new strains of Hantavirus.

5. Argentine and Bolivian hemorrhagic fevers

Among the other viral hemorrhagic fevers, only Argentine hemorrhagic fever caused by the Junin arenavirus and Bolivian hemorrhagic fever caused by the Machupo arenavirus, and both with their natural reservoirs in *Calomys sp.* and transmitted by aerosol, are characterized by kidney involvement in humans. Since these viruses cause a latent non-pathogenic infection in *Calomys*, with virus antigen in the lung and infectious virus in the lung and saliva, and since they cause acute fatal disease in newborn mice but latent infection in nude mice and older rats, they show many parallels to the Hantavirus genus of Bunyaviruses. Furthermore, the morphologic characteristics of the Hantaviruses have been noted to have arenavirus-like features (47-50), including variable size and grainy viroplasm. However, the tripartite, single-stranded RNA genome and the 3' terminal nucleotide sequence of the Hantaviruses indicate that they are Bunyaviruses. Yet, one wonders, in view of these biologic and structural similarities and the fact that they occupy very much the same ecologic niches in nature,

whether hantaviruses and arenaviruses may not have had a common evolutionary origin. Perhaps further sequencing and comparison of their genomes and subunit proteins may yield clues. It is important for HFRS workers to carefully follow the research developments of these two other airborne viral hemorrhagic fevers with chronic latent infection in their wild-rodent reservoirs.

Chapter II

WHO Collaborating Centre for Virus Reference and Research
(Haemorrhagic fever with renal syndrome)

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WHO Collaborating Centre for Virus Reference and Research

(Haemorrhagic fever with renal syndrome)

The World Health Organization (WHO) designated the Institute for Viral Diseases, Korea University, Seoul, Republic of Korea, as a WHO Collaborating Centre for Virus Reference and Research (Haemorrhagic fever with renal syndrome) on 16 August 1982.

The Centre agreed to the following :

1. Characterize Hantaan virus, the causative agent for Hemorrhagic fever with renal syndrome (HFRS) and standardize diagnostic test methods.
2. Maintain a repository of reference reagents.
3. Produce and distribute reference reagents.
4. Provide reference services.
5. Take an active part in collaborative studies on control measures.
6. Train scientists and produce training manuals.
7. Give consultant advice to national laboratories and develop surveillance of the infection of Hantaan virus in laboratory animals.
8. Report epidemiologic information on HFRS to WHO.

The Centre has been collaborating since 1980 with laboratories throughout the world on a seroepidemiologic survey of Hantavirus infections in humans and animals. The Centre has provided serologic diagnosis for suspected HFRS, supplied Hantaan and Seoul virus antigen preparations to many institutes, distributed reagents and protocols for standardization of techniques to be used for serologic

Table 1. List of hantaviruses adapted in Vero E6 cells at WHO Collaborating Centre for Virus Reference and Research (HFRS). The Institute for Viral Diseases, Korea University, Seoul.

A. Human strain :

1. ROK79 89	(blood)	Korea	7. US84 2	(serum)	Korea
2. ROK79 90	(blood)	Korea	8. ROK84 105	(serum)	Korea
3. ROK79 237	(blood)	Korea	9. Hubei 1	(serum)	China
4. LEE#188604	(blood)	Korea	10. Hubei 2	(serum)	China
5. ROK83 61	(blood)	Korea	11. Hubei 3	(serum)	China
6. ROK83 109	(serum)	Korea	12. Chen	(serum)	China

B. Apodemus mice strain :

1. 76 118	Korea	8. 83 15	Korea
2. 76 309	Korea	9. 83 18	Korea
3. 78 197	Korea	10. 83 23	Korea
4. 83 7	Korea	11. 83 27	Korea
5. 83 10	Korea	12. 83 125	Korea
6. 83 11	Korea	13. 83 138	Korea
7. 83 14	Korea	14. TCM 25 08 84	Yugoslavia

C. Urban rat strain :

1. 80 39 (#211808)	Korea	9. Thailand #605	Thailand
2. I RN 82 3	Korea	10 Brazil 2-4	Brazil
3. Girard Pt. #820132	USA	11. Hong Kong R 14	Hong Kong
4. Tchoupitoulas#401613	USA	12. Hong Kong R 19	Hong Kong
5. JTRN/82/17	Japan	13. Hong Kong R 35	Hong Kong
6. TR 352VE8	Japan	14. Hong Kong R 40	Hong Kong
7. Egypt R/12915	Egypt	15. Singapore R 36	Singapore
8. Egypt R/13120	Egypt	16. Sri R 87 1315	Sri Lanka

D. Laboratory rat strain :

1. KSNUSD84/30	Korea	3. SR-11 #191811	Japan
2. KSNUSD84/34	Korea	4. B-1 strain	Japan

E. Clethrionomys mice strain :

1. NE-Finn	Finland	3. RUV/38-83	USSR
2. USSR/CLS1/152	USSR	4. CG/18-20	USSR

F. Microtus mice strain :

1. Prospect Hill	USA

G. Hamster strain :

1. SNUS/Hamster 85/4	Korea

H. Bandicota indica strain :

1. Thailand #749	Thailand

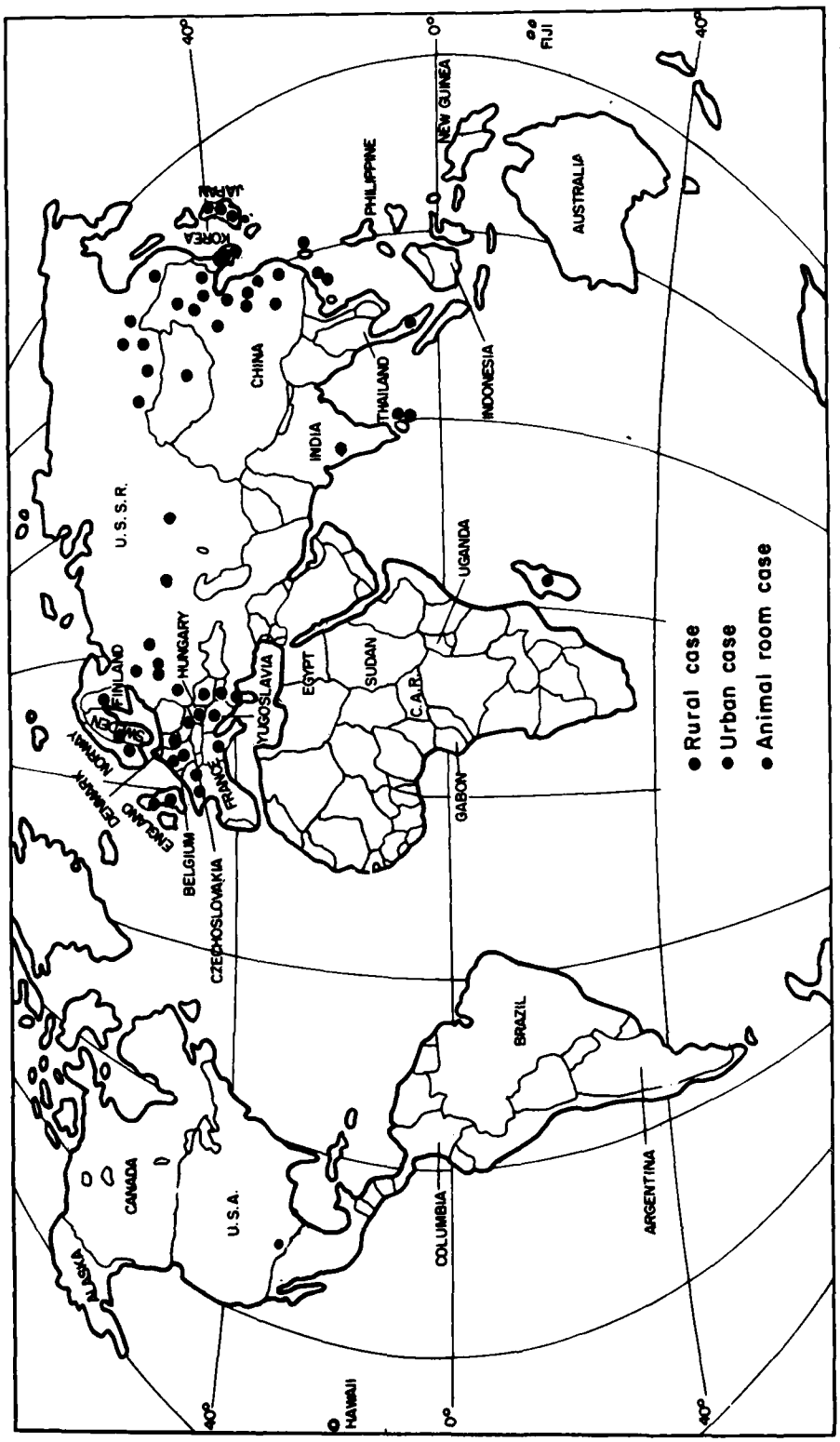

Fig. 1. Documented HFRS patients in the rural and urban areas, and in the animal rooms of institutes in the world.

diagnosis of HFRS to participating laboratories according to WHO's recommendation and trained scientists from many countries in Asia, including Japan, India, China, Sri Lanka, Hong Kong, Malaysia and Singapore. The Centre has 53 strains of Hantaviruses including prototype strains of Hantaan (51), Seoul (52), Puumala (53) and Prospect Hill virus (54), adapted to grow in Vero-E6 cells that were isolated from HFRS patients and from rural, urban and laboratory rats from many parts of the world, as shown in Table 1 and is ready to provide reagents as requested.

Fig. 1. shows world-wide distribution of HFRS patients. There are about 200,000 documented HFRS patients with 3–10% fatality every year in the world as shown in Table 2.

At a WHO sponsored meeting in Tokyo, 22-24 February, 1982, the working party on HFRS recommended that the KHF-like diseases with different names should be referred to as "Haemorrhagic fever with renal syndrome (HFRS)".

The etiologic agent of KHF was first isolated from lungs of *Apodemus* mice caught in Songnaeri, the endemic area of KHF (Fig. 2), near Hantaan river and was named as Hantaan virus after Hantaan river (Fig. 3) which runs near 38th parallel between North and South Korea.

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Table 2. Documented number of hospitalized haemorrhagic fever with renal syndrome patients in the world.

Country	Number of HFRS patients
Asia	
China	100,000–150,000 per year
Korea	500–2,000 per year since 1951
Japan	316 (1964–1987)
U.S.S.R.	11,000 (1978–1983)
Hong Kong	7 (1985–1987)
Malaysia	6 (1985)
Sri Lanka	4 (1987)
Europe	
Finland	609 (1980–1985)
Sweden	hundreds since 1954
Denmark	hundreds since 1957
Bulgaria	406 (1953–1982)
Hungary	136 (1952–1980)
Yugoslavia	hundreds per year since 1952
France	102 (1982–1987)
Germany	8 (1984–1987)
Italy	14 (1984–1987)
Greece	37 (1982–1987)
Belgium	4 (1987)
Great Britain	7 (1984–1987)
Americas	
U.S.A.	1 (1986)
Africa	
Madagascar	10 (1986–1987)

Fig. 2. The photograph of Songnaeri village nearby Hantaan river where Hantaan virus was first isolated from *Apodemus* mice caught in this endemic area of HFRS in 1976.

Fig. 3. The picture of Hantaan river which runs near the 38th parallel between North and South Korea.

Chapter III

Clinical Manifestations of HFRS

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1. Korean hemorrhagic fever (Hantaan virus infection)

To assist readers in diagnosing Korean hemorrhagic fever (KHF) type of HFRS, specific historical data, clinical manifestations, and the course of the disease are presented.

Based on time of entry of personnel into endemic areas, the incubation period for KHF is usually 2 to 3 weeks, varying from 5 to 42 days. Clinically, the infection is characterized by acute disease with sudden onset of fever with chills lasting 3 to 6 days, conjunctival injection, prostration, anorexia, vomiting, abdominal pain, hemorrhagic manifestations which begin about the third day, proteinuria about the fourth day, and hypotension about the fifth. Renal disorders, varying from mild to acute renal failure, are characteristic during the acute phase and may persist for several weeks.

The overall mortality rate was 7% to 15% in the 1960s, but this has recently dropped to less than 5% due to earlier diagnosis and more intensive care (55, 56). Residual complications during recovery are now rare.

A. Clinical manifestation and course of disease

KHF can be divided into five phases : febrile, hypotensive, oliguric, diuretic and convalescent, according to its characteristic clinical, laboratory and pathophysiologic features (Table 3). However, some patients may present with overlapping phases (55, 57, 58, 59).

Between 20% and 30% of patients exhibit severe courses of shock, serious hemorrhage, marked electrolyte imbalance, renal failure, and pulmonary edema, while 30% to 40% of patients have mild courses. Between 50% and 60% of all cases exhibit a moderate course (60).

Table 3. Haemorrhagic fever with renal syndrome
(Korean hemorrhagic fever by Hantaan virus infection)
An acute disease characterized by fever, prostration, vomitting, a variety of hemorrhagic manifestations, shock, proteinuria and renal failure.
Mortality is 5-10%

Phase	Observation	Clinical course: Criteria for severity		
		Mild	Moderate	Severe
Febrile	Max. temperature C	38-39	40	≧41
	Days temp. over 38 C	1-5	6	≧ 7
	Petechiae, flush	0	1 3 -	4 ·
	Max. WBC count	≦14,000	14,000-30,000	≧30,000
Hypotensive	Max. hematocrit	to 50	51 56	≧57
	Min. systolic B.P.	96-120	81 95	≦80
	Hours of hypotension	24	24 47	≧48
	Min. platelets	≧90,000	40,000 89,000	≦39.000
Oliguric and diuretic	Min. hematocrit	≧45	35 44	≦34
	Max. systolic B.P.	≦140	141 169	≧170
	Days of hypertension	1	1 2	≧2
	Max. BUN	20-79	80 149	≧150
	Days of proteinuria	<4	4-5	≧5
	Max. daily urine vol.	≦3,400	3,500 4,900	≧5,000
Convalescence	Days to recover Urine S.G. 1,023	≦33	34 54	≧55

(1) Febrile phase

The febrile phase usually lasts 3 to 8 days with sudden onset of high fever to 40 C, chills, general malaise, weakness and generalized myalgia. This may be followed by severe anorexia, dizziness, headache and eyeball pain. The extensive retroperitoneal or peritoneal edema resulting from extravasation of plasma due to increased capillary permeability is responsible for the severe abdominal and back pain and tenderness of the renal area. The characteristic findings of KHF, which are flushing over the face, neck and anterior

chest and injection of the eyes, palate and pharynx, also occur in the febrile phase. Toward the end of this phase, conjunctival hemorrhage occurs and fine petechiae are observed in the axillary folds, face, neck, soft palate and anterior chest wall (Fig. 4 and 5).

Generally, certain clinical features appear to correlate closely with the prognosis and the severity of the disease. These include the degree of facial flushing, fever, conjunctival hemorrhage, duration of fever, number and degree of petechiae and presence of facial petechiae. The white blood count can be either normal or elevated. During the febrile phase, urine may contain a small amount of albumin, which then increases abruptly in the late period, becoming massive proteinuria in the majority of cases.

(2) Hypotensive phase

The hypotensive phase develops abruptly and can last from several hours to 3 days. The classical signs of shock can be noted, including tachycardia, narrowed pulse pressure, hypotension, cold and clammy skin and dulled sensorium, even confusion. In mild cases, blood pressure returns to normal some hours later ; but in severe forms of HFRS, hypotension manifests as clinical shock and one third of deaths are associated with irreversible shock in this stage. In moderate cases, recovery usually occurs within 1 to 3 days if prompt and effective treatment is given.

The decrease in cardiac output (hypotension or shock), which constitutes the basis for the hypotensive phase of HFRS, is attributable to decreased effective circulating blood volume because of capillary leakage of plasma and dilatation of capillary and venular beds. The clinical symptoms of the febrile phase frequently persist in aggravated form into the hypotensive phase ; and thirst, restlessness, or even coma occasionally develop. Laboratory data show marked proteinuria, mild hematuria and falling urinary-specific gravity. Hematocrit increases, a leukemoid reaction occurs and the number of platelets decreases. Capillary hemorrhages

are most prominent at this time. Oliguria may appear during the late shock phase and then blood urea and creatinine begin to rise.

The thrombocytopenia has been related to disseminated intravascular coagulation with associated multiple defects in the first and second stages of coagulation, hypofibrinogenemia and accumulation of fibrinogen degradation products.

(3) Oliguric phase

The oliguric phase usually lasts from 3 to 7 days. Blood pressure begins to return to normal, but many patients (up to 60%) become hypertensive due to their relative hypervolemic state. Patients may have severe nausea and vomiting associated with persistent oliguria, but facial flushing and petechiae begin to diminish.

The tendency to bleed becomes more marked and various combinations of epistaxis, conjunctival hemorrhage, cerebral hemorrhage, gastrointestinal bleeding and extensive purpura are shown by about one third of patients.

The presenting picture is usually that of an oliguric patient who has a rising concentration of blood urea and creatinine. Hyperkalemia, hyponatremia and hypocalcemia may also be present and metabolic acidosis is occasionally seen. Central nervous system symptoms and pulmonary edema occur in severe cases. About 50% of the fatal cases occur during the oliguric phase (60, 61, 62).

(4) Diuretic phase

Clinical recovery begins with the onset of the diuretic phase, which lasts for days or weeks, but diuresis may be delayed in some patients due to dehydration, electrolyte imbalance, or infection. Brisk diuresis of low specific gravity urine may be due to further residual tubular damages and diminished tubular reabsorbtion.

A diuresis of 3 to 6 liters daily is the rule. Vasopressin injection (Pitressin) and fluid restriction have little or no effect on diuresis. The daily urine volume and the duration

Fig. 4. Classic picture of KHF in a 22 year old man 7 days after the onset of fever. Note the flushed face, conjunctival hemorrhage.

Fig. 5. Petechiae in the axillary region of a patient 6 days after onset of fever.

of the diuretic phase are greatly influenced by the severity of the disease.

(5) Convalescent phase

The convalescent phase takes 2 to 3 months ; and while mild anemia may persist, recovery is the rule. This phase is characterized by a progressive recovery of the glomerular filtration rate. Renal blood flow and urinary concentration ability are restored up to 70% of normal status within 3 months of the illness. But unusual complication, such as neurologic sequela fallowing CVA, hypopituitarism or chronic renal failure was rarely experienced.

B. Clinical diagnosis

There is no specific clinical test to confirm HFRS, so physicians must interpret abnormal laboratory data-changes in urine, CBC, serum electrolytes, and blood chemistry-together with clinical manifestations.

The clinician makes an initial diagnosis on epidemiologic and clinical grounds, which is confirmed by specific serologic tests that demonstrate increased specific immunofluorescent antibodies against Hantaan virus in sera collected twice during the course of the illness at 1-week or greater intervals. Immunofluorescent antibodies appear during the first week of symptoms, reach a peak at the end of the second week, and persist as long as 36 years.

For the diagnosis of HFRS, all signs and symptoms are not pathognomonic. A history of exposure in an endemic area, particularly during an epidemic season (late autumn), and appearance of fever, abdominal pain and severe retching, marked proteinuria, flush, shock, hemorrhagic diathesis, pulmonary edema, associated with leukemoid leukocytosis, thrombocytopenia, hemoconcentration and azothemia are virtually diagnostic. In study of 12,398 clinically suspected HFRS cases in Korea for 10 years, 44% were confirmed by serology (63).

Differential diagnosis should be considered with the following diseases in Asia : hemorrhagic scarlet fever, leptospirosis, scrub typhus, dengue, Henoch-Schoenlein's purpura, non-A,non-B hepatitis, hemorrhagic glomerulonephritis, septicemia, thrombocytopenia, endocarditis, heatstroke, disseminated intravascular coagulation (DIC), and acute renal failure (other causes).

C. Treatment

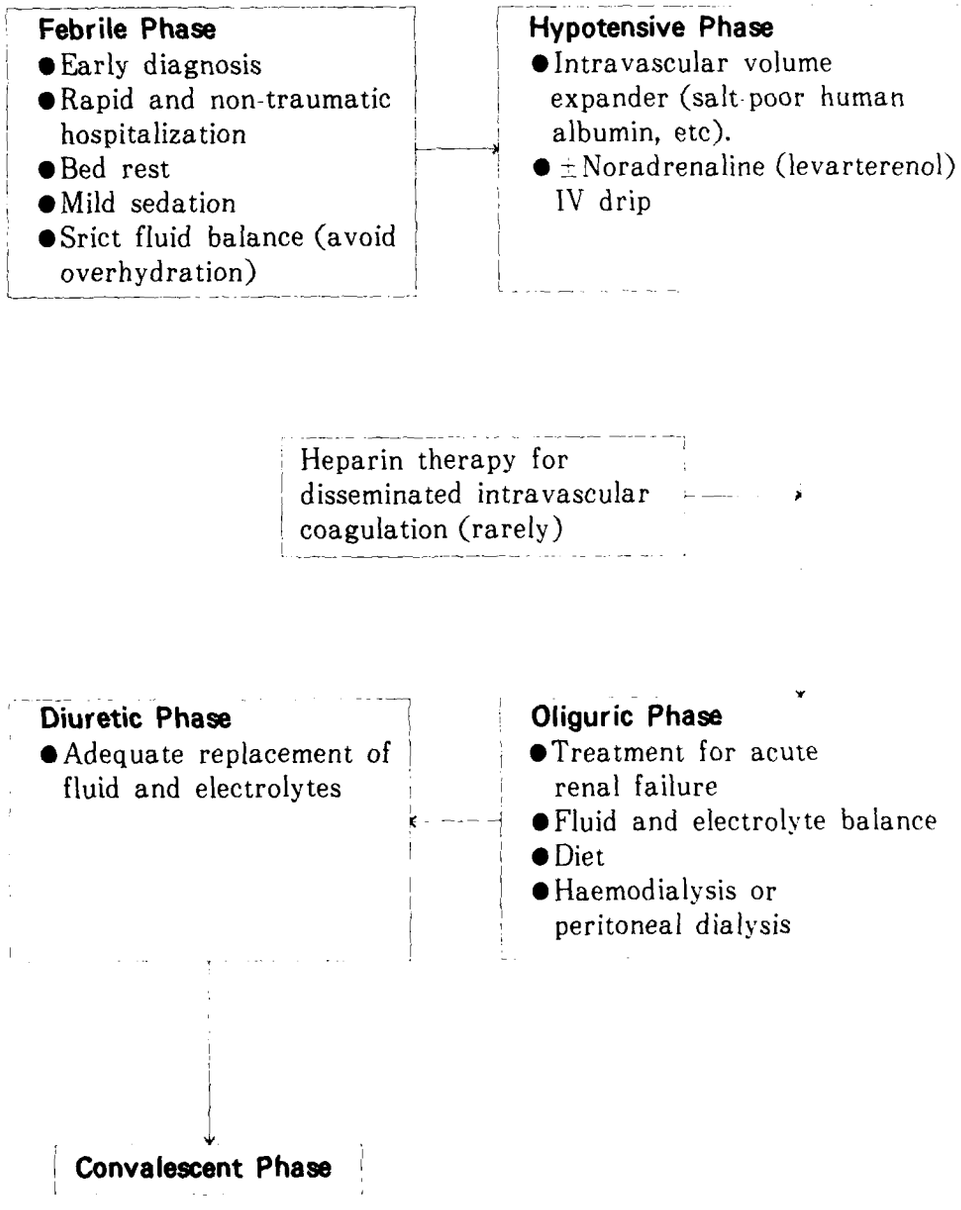
There is no specific treatment of HFRS, so the management of the patient must be supportive and based on an understanding of the pathophysiologic characteristics of the disease and on an evaluation of clinical and laboratory findings (Table 4). Early diagnosis and hospitalization before the onset of the hypotensive and hemorrhagic phenomena are very important. Therefore, transportation to a hospital should be as rapid and nontraumatic as possible.

The febrile phase demands bed rest, mild sedation, analgesics, and strict maintenance of fluid balance, especially avoiding overhydration. If a normal water balance can not be maintained, dehydration is preferable to overhydration.

Treatment in the hypotensive phase requires administration of an intravascular volume expander, such as salt-poor human albumin infusion with other general measures for shock. Dextrose solution, saline solution, or other electrolyte solutions are not fully effective and can cause overhydration and thus should not be used. Continuous intravenous drip of noradrenaline is helpful when shock is not reduced with large amounts of volume expander. Whole blood transfusion is contraindicated when the patient's hematocrit is elevated. If disseminated intravascular coagulation is evident, heparin therapy is sometimes helpful, but it should be administered with caution.

Treatment in the oliguric phase is the same as for acute renal failure. Fluid should be restricted sufficiently to effect a loss of approximately 0.5 K body/day. In general, this

Table 4. Management of Korean hemorrhagic fever



amounts to the administration of 400 to 500 ml of fluid plus the amount lost in the urine and gastrointestinal tract. If the patient is able to take food by mouth, a diet providing approximately 2,000 calories, which is kalium and protein free and essentially natrium-free, is recommended.

If parenteral alimentation is necessary, the usual treatment is to infuse dextrose (at least 100 g/day) and vitamins in hyperosmotic solution because of the requirement for fluid restriction. Precautions are required to prevent venous thrombosis and infection of the venous catheter.

Particular attention may be necessary to prevent the development of progressive hyperkalemia.
The administration of a kalium-binding resin either by mouth or by enema will generally prevent serious elevations of plasma kalium concentration. If marked hyperkalemia is present, intravenous glucose and insulin, natrium bicarbonate, or calcium chloride will all protect temporarily against the cardiotoxic effects of kalium excess.

In cases of hyperkalemia, pulmonary edema, or severe uremic symptoms, dialysis is the most effective treatment, and either peritoneal or extracorporal hemodialysis may be employed. When frequent dialysis is employed, only modest salt and water restriction is needed, and a 2,000-calorie diet may be allowed. Thus, for patients who are expected to have a clinically severe disease course, early and frequent dialysis is recommended.

Sometimes during the late oliguric and early diuretic phases, severe hypertension develops, requiring the use of antihypertensives or even phlebotomy. In the diuretic phase, attention must be paid to adequate replacement of fluid and electrolytes.

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2. Nephropathia epidemica (Puumala virus infection)

The Nephropathia epidemica (NE) type of hemorrhagic fever with renal syndrome (HFRS) has been known since 1934 (64, 65) when it was described in Scandinavia and Europe. The name, nephropathia epidemica (66), indicates that it is not considered to be a hemorrhagic fever, but rather a renal disease, although both clinical comparisons (29, 67) and serologic evidence (68) justify its belonging to the HFRS complex.

The purpose of this chapter is to give advice on how to diagnose cases of NE. This is more difficult than diagnosing more severe types of HFRS because NE is clearly a milder disease in which the renal manifestation dominates the hemorrhagic features (Table 5). With this exception and that in mortality, 10% in KHF versus 0.2% in NE, both types are clinically very similar. The use of three of five phases of disease in which KHF has been divided has not proven very useful in diagnosing NE because they are not easily distinguished in mild and moderate cases, which form about 90% of all NE cases.

A. Clinical symptoms

The basic combination of symptoms, signs, and laboratory findings to create the clinical suspicion of NE is presented in Table 5. In addition to these basic findings, NE has other manifestations that can be useful in diagnosing NE. They are presented in Table 6, which also contains the most prominent findings in isotope renography (nephrography) and renal biopsy. Experience has shown that the details of Table 5 added with examinations on hemoconcentration and thrombocytopenia and isotope renography are most useful in diagnosing NE (Table 7).

Table 5. Marked clinical difference between NE and KHF types of HFRS.

Clinical symptoms, and findings	NE (29) Percentage incidence	KHF (69) Percentage incidence
Hemorrhagic manifestation		
Conjunctival injection	18	97
Petechial rash	12	95
Petechiae in throat and soft palate	36	98
Purpura	0	37
Hematoma	0	5
Hematemesis and melena	0	3
Respiratory and circulatory symptoms and signs		
Cough	6	31
Dyspnea	0*	25
Pneumonic infiltrations	0	5
Pulmonary edema and/or hemorrhages	0	7
Shock	0*	10
Neurologic symptoms		
Dizziness	12	94
Myopia (blurred vision)	12	52
Convulsions	0	9
Others		
Enlargement of cervical lymph nodes	15	41
Prodromal signs	8	68
Secondary infections	0*	33
Mean peak levels of WBC	12,000/mm^3	20,000 mm^3
Mean of minimum platelet counts	50,000–100,000/mm^3	10,00–20,000 mm^3

*Or very few.

The pattern of renography is not pathognomonic to NE, because it appears also in other acute tubular and interstitial nephritides, but it is common and clear in NE and thus useful. The most prominent finding in a renal biopsy study is interstitial hemorrhage, which is also not pathognomonic but is common and clear.

Table 6. Clinical diagnosis of typical cases of NE.
a. The complex of basic clinical symptoms, signs and laboratory findings (29).

Clinical symptoms, signs and laboratory findings	Percentage incidence	Mean time of occurrence (Day of fever is counted as the first day)	Special features
Fever	100	1st to 5th day	Sudden in 65% over 39 C
Headache	90	2nd to 6th day	Intensive in 30 % of the cases
Backache	82	4th to 9th day	
Abdominal pain	67	4th to 9th day	
Nausea and/or vomiting	75	3rd to 6th day	
Petechiae in throat and soft palate	36	3rd to 6th day	Slight
Facial flush	32	3rd to 6th day	
Petechial rash	12	3rd to 7th day	Slight
Proteinuria	100	3th to 10th day	Unselective, peak usually 3 g l, may be up to 20 g l
Microscopic hematuria	74	4th to 10th day	
Leukocyturia	67	4th to 10th day	
Azotemia	86	4th to 15 day	
Oliguria (100-400 ml/24 hr)	54	3rd to 10 day	
Anuria (below 100 ml/24 hr)	8	3rd to 7th day	
Polyuria (over 2,000 ml/24 hr)	97	7th to 17th day	Mean of peak 3,500 ml 24 hr, may be up to 6,500 ml 24 hr
Decrease of renal concentration capacity (measured by specific gravity of morning urine below 1,024)	100	4th day to 2nd - 6th month	

The diseases to be considered in differential diagnosis of NE depend on the local pattern of diseases. Thus, is seems useful to present the list of diseases in Scandinavia and the most prominent features differentiating them from NE (Table 8).

Table 7. Clinical diagnosis of typical cases of NE.
b. Additional clinical findings (29).

Clinical symptoms, signs and laboratory findings	Percentage incidence	Mean time of occurrence (Day of fever is counted as the first day)	Special features
Circulatory findings			
Hypotension	40	3rd to 11th day	Reduction of systolic pressure by 15-20 mm Hg
Bradycardia	36	8th to 13th day	Below 60 beats min
ECG changes	42	5th to 15th day	Mainly T-wave changes, ST changes, and prolongation of P-R and P-Q times
Neurologic findings			
Restlessness	25	3rd to 6th day	
Amnesia	14	3rd to 6th day	Duration 1-3 days
Myopia	12	4th to 8th day	Duration 1-5 days
Aggressiveness	9	3rd to 6th day	
Elevation of proteins in CSF	29	4th to 9th day	
Hematologic findings			
Hemoconcentration	52	3rd to 8th day	2-3 g/100 ml
Thrombocytopenia	60-80	3rd to 14th day	6-10 days, lowest value has been 20,000 mm^3
Leukocytosis	57	4th to 10th day	Highest value has been 38,000/mm^3
Changes in serum enzymes			
Elevation of serum LDH	71	3rd to 10th day	
Elevation of serum ASAT	52	3rd to 16th day	
Elevation of serum ALAT	40	3rd to 18th day	
Elevation of serum CPK	27	3rd to 10th day	
Changes in serum protein			
Reduction of albumin	88	5th to 23rd day	
Elevation of α^2-globulin	56	3rd to 20th day	
Elevation or IgM	85	3rd day to 3rd month	
Elevation of IgG	54	4th day to 2nd month	

Miscellaneous			
ESR over 10 mm/hr	100	4th day to 2 week	Mean 50 mm/hr highest value has been 118 mm/hr
Reduction of serum sodium	73	4th to 19th day	
Metabolic acidosis	40	4th to 19th day	
Most prominent findings in special examinations			
Isotope renography (nephrography)			
Retention change	96	3rd day to 3rd month	
Renal biopsy			
Hemorrhages into the medullary interstitium	90-100	3rd day to 3rd month	
Inflammatory cell infiltration in medullary interstitum	95	5th to 21st day	
Inflammatory cell infiltration in cortical interstitium	90	3rd day to 5th month	

Table 8. Most common diseases in differential diagnosis of NE in Scandinavia, in order of prevalence.

Disease	Most useful differentiating featutes
Acute respiratory infection (e.g., influenza)	NE has none or only slight respiratory symptoms and signs but has azotemia
Acute febrile UTI	NE has no significant bacteriuria
Bacterial sepsis	NE has no bacteremia
Acute glomerular nephritis	NE has more rapid transitory proteinuria and azotemia
Chronic renal disease (glomerular or interstitial) in connection with acute infection	NE has usually no earlier renal disease in anamnesis
Acute meningitis (bacterial or viral)	NE has only slight pleocytosis and no bacteria in CSF
Acute appendicitis or stomach perforation	NE has proteinuria and azotemia
Salmonellosis	NE has negative stool and blood cultures
Leptospirosis	NE has no bacteriological and antibody finding
Other acute infectious tubular and interstitial nephritis	Most difficult to differentiate. More accurate comparison to clinical pattern of NE is useful. Serologic diagnosis necessary

B. Treatment of NE

Nephropathia epidemica is a viral disease that is generally mild and needs no particular treatment. No special treatment has been available against the primary viral etiology. Because of the mildness of dlisease the need of it is not so obvious as in KHF type. Primary hospitalization, however, is preferred for the correct clinical diagnosis and differential diagnosis in the acute phase of disease when serologic results of paired sera are not yet available. It is also easier and quicker in the hospital to verify complications needing therapy, including :

(1) Uremic syndrome needing hemodialysis or peritoneal dialysis in 1% to 3% of the cases.
(2) Secondary bacterial or fungal infections needing chemotherapy in 1% to 2% of the cases.
(3) Derangements in water, electrolyte, and acid-base balance (other than those needing dialysis therapy) needing parenteral therapy in 15% to 20% of the cases.
(4) Discomforts like headache, backache, abdominal pain, vomiting, and psycho-organic symptoms needing analgesics, antispasmodics, or tranquilizers in 50% to 70% of the cases.

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3. Hemorrhagic fever with renal syndrome (Seoul virus infection)

Many urban cases of HFRS in Korea, China, Japan and Southeast Asia, and laboratory infections in many parts of the world are caused by Seoul virus (14, 15, 16, 24, 25, 26, 27, 28, 70-76). Urban commensal rats (*Rattus norvegicus* and *Rattus rattus*) and laboratory rats are main reservoir hosts and they transmit the disease to man. Some urban cases and laboratory cases are severe ; however, many of the cases are milder than Hantaan virus infection. In general, five phases of the disease are shorter than classic type of KHF and sometimes it is difficult to recognize phases of the disease clearly. The clinical characteristics of the disease are high fever, fatigue, anorexia, vomiting, backache, myalgia, abdominal pain, conjunctival injection, petechiae on soft palate and hepatomegaly. Laboratory abnormalities include proteinuria, microscopic hematuria, lymphocytosis, thrombocytopenia, increase in GOT, GPT and transient glycouria. These findings are based on observation of 56 cases of Seoul virus infection in Korea and Japan (75, 76). The most characteristic manifestations are strong abdominal symptoms, hepatomegaly and hepatic dysfunction, and mild renal dysfunction. A comparison of clinical features of HFRS caused by infection of different serotypes of Hantavirus in the world is shown in Table 9.

Recently, we have documented three cases of HFRS in Hong Kong and two cases of HFRS in Malaysia, tropic areas where the disease is not known to exist (77, 78). Serologic confirmation of the diagnosis of HFRS was obtained by the demonstration of significant antibody rises to Seoul virus in the patient's acute and convalescent phase sera by IFAT, ELISA test and PRNT.

Table 9. Comparison of clinical features of HFRS in various countries of Euro-Asia.

	Hantaan virus infection		Seoul virus infection	Puumala virus infection
	Korea[69,80]	China[81]	Japan[75] & Korea[76]	Finland[29]
Fever	100%	100%	100%	100%
Anorexia	96		96	70
Chills	92		70	60
Nausea	82	72	61	78
Vomiting	63	58	45	70
Myalgia	78	69	52	20
Headache	86	83	42	90
Abdominal pain	23	25	65	67
Backache	95		80	82
Constipation	60		37	34
Diarrhea	11	37	24	12
Dizziness and vertigo	100	41	7	12
Ophthalmalgia		34	15	0
Blurred vision		18		12
Conjunctival injection	64	23	79	18
Pharyngeal or palatal injection	55	64	79	36
Petechiae on body	32	56	31	12
Hemorrhages (epitaxis, melena, hematemesis etc)	72	31	26	10
Hepatomegaly	7		15	0
Lymphadenopathy	38	3	15	15
Preorbital edema	9	17	4	56
Proteinuria	96		94	100
Hematuria	85	86	73	74
Oliguria $<$500 ml	67	59	37	54
Polyuria $>$2,000 ml	92	87	63	97
Leukocytosis $>$100,000 mm^3	91	92	41	57
Thrombocytopenia $<$100,000 mm^3	54	78	70	80
Increased ESR $>$20 mm/h	72		7	90
BUN $>$or serum Cr $>$2 mg dl	94	100	50	70
Hypotension ($>$90/60 mm Hg)	80	42	22	40

80 ; Counts and Seltser, 40 cases (1953). 69 ; Lee, et al. 125 caess (1980).
81 ; Cohen, et al. 71 cases (1981). 76 ; Lee, et al. 29 cases (1987).
75 ; Morimoto, et al. 27 cases (1985). 29 ; Laehdevirta, 76 cases (1971).

Clinical details of 3 HFRS patients who were diagnosed clinically as having hepatitis, thrombocytopenia and proteinuria in Hong Kong all included fever, chills, jaundice, thrombocytopenia, abnormal liver and renal functions and proteinuria.

Clinical features and selected laboratory data of 2 HFRS patients who were diagnosed clinically as Dengue and leptospirosis in Malaysia are not typical of the disease seen in endemic regions of HFRS. Renal involvement, which is characteristic of HFRS, was mild and the predominant symptom was a persistently marked elevation of serum transaminases suggestive of hepatitis. The patient developed a petechial skin rash and had a severe thrombocytopenia.

Recently, mild form of HFRS was demonstrated serologically among non-A, non-B hepatitis patient in Korea (79). IgM antibodies to Seoul virus were found only in 4 out of 60 hepatitis patients and the titers of IgM antibodies were over 800 units. In 2 of them, IgM antibodies persisted in high titers over 1 year.

It is suggested that Seoul virus should be considered as one of the etiologic agents causing non-A, non-B hepatitis in Korea and other parts of the world as well.

Chapter IV

Epidemiology

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Epidemiology

It was long believed that Hemorrhagic fever with renal syndrome (HFRS) only occurred in rural areas, with farmers and soldiers being the most likely victims. Areas infected with Hantaan virus were also thought to be limited to certain locations, infecting only their inhabitants and travelers. Recent findings (70, 82-88), however, have shown that HFRS patients can be found throughout the world.

1. Distribution

Seroepidemiologic surveys and documented case reports show that Hantaviruses are widely distributed throughout much of the world, as demonstrated by the presence of antibodies against the agents in sera from humans, field rodents, urban rats, and laboratory rats (83-89). Global distribution of HFRS patients, field rodents, urban rats, and laboratory rats infected with Hantaviruses is shown in Fig. 6.

Antibodies against Hantaan or Seoul viruses were detected in human and urban rat sera in the Americas (Alaska, Argentina, Brazil, Canada,. Columbia, United States, including Hawaii), the Western Pacific and Southeast Asia (Burma, Fiji, Hong Kong, India, Malaysia, Sri Lanka, Singapore, New Guinea, Philippines, Taiwan, Thailand), as well as in Africa (Central African Republic, Egypt, Gabon, Nigeria, Sudan, Madagascar, Uganda) where HFRS is not known to exist (88) as shown in Fig. 6.

There are at least two froms of the disease : mild (29) and severe (70). The severe from is common in Asian countries. In the Republic of Korea, several hundred cases occur annually in rural and urban areas with a case fatality rate

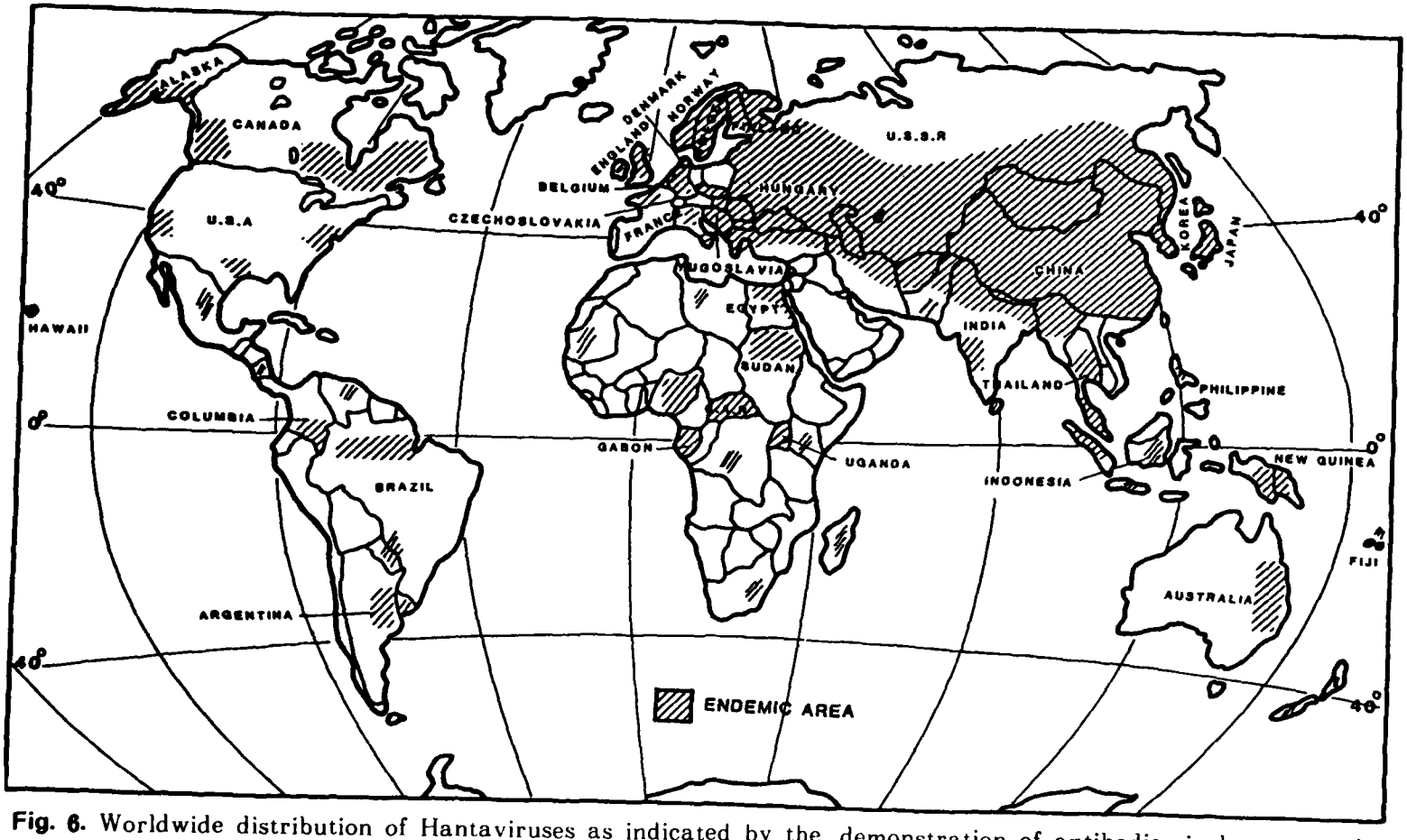

Fig. 6. Worldwide distribution of Hantaviruses as indicated by the demonstration of antibodies in humans and rodents.

of approximately 5%. In Japan by the end of 1985, 126 cases with 1 death have been reported since 1969, the origin of infection being laboratory rats infected with Seoul virus in the animal rooms of medical centers and institutes (73). In the 1960s, approximately 150 patients were hospitalized in Osaka city with 3 deaths (16). In China, about 100,000 hospitalized cases were reported in 1984 and 120,000 cases in 1985, with a 7% to 15% case fatality rate. Mild forms of the disease were reported in some cities. In Russia, about 11,000 cases of the disease have been reported during the past 5 years (86).

The majority of cases in Europe are mild. Several hundred cases of hemorrhagic fever with a syndrome similar to mild form of the Korean disease have been reported annually as nephropathia epidemica in Scandinavia (Finland, Norway, Sweden), and as epidemic nephritis in Eastern Europe (Bulgaria, Czechoslovakia, Hungary, Romania, Yugoslavia). Several cases of HFRS have been reported in England, Germany, Belgium, France, and Greece (26, 28, 86, 88, 89).

2. Epidemiologic type

There are three epidemiologic patterns of HFRS according to the location of outbreak and the reservoir host of the disease (70, 89) : rural, urban, and animal room.

A. Rural type

The reservoir of HFRS in the rural endemic areas of Korea is the field mouse, *Apodemus* species (3, 4, 90). These rodents live only in fields but will invade houses during the snow season. Rural type cases occur all year around in the endemic rural areas. There are two seasonal peaks in late spring and fall when incidences of the infectd *Apodemus* mice are high. Victims are primarily farmers and soldiers, ranging in age from 20 to 50, working or stationed in the field. The rural type has been well known for the last 50 years (Fig. 7).

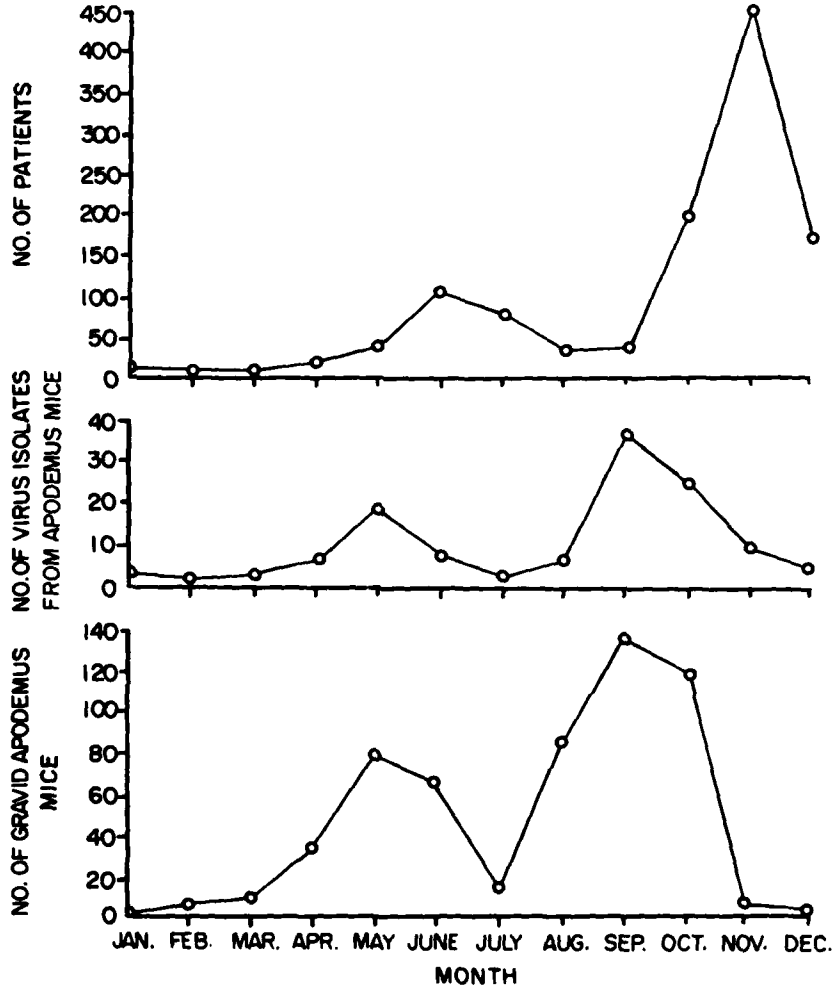

Fig. 7. Cumulative seasonal prevalence of HFRS, infected *Apodemus agrarius* with Hantaan virus and of gravid *Apodemus agrarius* in the endemic area of Kyunggi province, Korea, 1975–1978.

The European HFRS is also of a rural type, and the reservoir is *Clethrionomys glareolus* in Scandinavia (11). There is increasing evidence that many species of wild rodent and insectivora are also reservoirs of Hantaviruses in Russia and China (97-100).

B. Urban type

The reservoir for the urban type of HFRS is house rats. This type threatens to endanger the world as never known before. Recently, over 100 cases of HFRS were reported in metropolitan areas of Seoul and other large cities in Korea annually where the patients had never been outside the city limits, but who had histories of contact with house rats (63). 130 cases of HFRS were also reported among residents of urban areas of Osaka, Japan, during the 1960s. Cases of the urban type occur throughout the year, but tend to be more frequent in fall and winter seasons (73).

C. Animal room type

The reservoir for animal room HFRS is colonized experimental rats. This was proven by demonstration of antibody and isolation of the virus from experimental rats (24-28, 91-94). There were 33 outbreaks of HFRS from 1976 to 1985 among personnel in medical center animal rooms in Korea and Japan where Hantaan or Seoul virus experiments had not been conducted. The victims numbered 164, one of whom died. Animal room HFRS may occur at any time of the year, but a series of outbreaks occurred during the winter season in non-ventilated animal rooms when the air in the rooms was dry. Laboratory infections with Hantaan-related virus where Hantaan-related virus experiments have never been conducted have occurred not only in Asia but also in Europe. A few cases of HFRS occurred in animal rooms of research institutes in Belgium and Great Britain (26, 28, 94). These incidents demonstrate that exports, imports, and exchanges of special animal models of infected rats among research institutes are very dangerous.

3. Transmission

Large quantities of virus are excretd in the saliva, urine, and feces of infected mice, *Apodemus agrarius* (90) and

Clethrionomys glareolus (95). Excretion of Hantaan virus in *Apodemus* mice persists in the saliva and feces for at least 1 month and in the urine for 12 months.
Horizontal transmission of the virus among *Apodemus* mice has been demonstrated. In these experiments, *Apodemus* mice were infected by inoculation. Noninfected *Apodemus* mice caged with the infected *Apodemus* mice for several days acquired infection beginning 10 days after initial exposure. Results were the same when ectoparasitized and clean animals were used in these experiments. The main route of infection in *Apodemus* mice is via the respiratory tract, although it can be transmitted via the saliva, urine or feces.
Transmission of the virus among house rats and experimental rats was almost same as *Apodemus* mice but excretion of the virus in excreta was shorter, about 1 2 weeks (96).
There is no evidence of direct human-to-human transmission of the virus in hospitals.

A. Reservoirs

The reservoir of Hantaan virus in the rural endemic areas in the Republic of Korea is *Apodemus agrarius coreae (Fig. 8)* ; in Finland and west of the Ural mountains, the reservoir of Puumala virus is *Clethrionomys glareolus* and field mice ; and in the urban areas of the Republic of Korea, Japan, and China, *R. rattus* and *R. norvegicus* (Fig. 9) are reservoirs of Seoul virus. Hantavirus antigens were detected in 16 different rodent species and 4 different insectivorous species in the USSR and China (97-100). Colonized experimental rats, a dangerous reservoir of Hantaan and Seoul viruses, have been responsible for several outbreaks of HFRS among laboratory animal room personnel at research institutes in Belgium, China, Japan, Great Britain, and the Republic of Korea.

B. Vectors

The identification of Hantaan virus as a member of the family Bunyaviridae suggests that it may be arthropod-transmitted, but that role has not been confirmed.

Fig. 8. *Apodemus agrarius*, the reservoir host of Hantaan virus in Korea, China and USSR.

Fig. 9. *Rattus norvegicus*, the reservoir host of Seoul virus in many parts of the world.

It has been hypothesized that Hantaan virus can be transmitted by ectoparasites harbored by various field mice (37). Until now, Hantaan virus has not been isolated from arthropods nor has it been successfully cultivated in tissue culture cell lines of arthropods.

4. Groups at risk

The disease appears to most frequently affect persons 20 to 50 years old ; cases in children under 10 years old are rare. Although the disease occurs in both sexes, the figures accumulated show a significantly higher prevalence in males. The victims are primarily farmers and soldiers working and on duty at field stations.

The following groups of personnel are also at increased risk of infection by Hantaviruses :

(1) Health personnel working in laboratories where research into the disease is being conducted.
(2) Animal room workers.
(3) Animal breeders.
(4) Rodent control officers.
(5) Biologists.
(6) Soldiers.
(7) Farmers.
(8) Hunters and camping people.

Chapter V

Clinical and Epidemiologic Sampling

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Clinical and Epidemiologic Sampling

1. Clinical sampling

HFRS with it diverse clinical symptoms occurs in many parts of the world ; however, it is impossible in the individual case with moderate to mild clinical symptoms to diagnose Hantaan and related virus infections on clinical manifestations alone. Even in severe cases, a correct clinical diagnosis is made in only about 50% of seropositive Korean patients (101, 102). The symptoms of infection with Hantaan ralated viruses in many parts of the world where HFRS is not yet known to exist may be mild or even different from typical forms of KHF and nephropathia epidemica. A serologic survey of Hantavirus infection in humans would demonstrate distinct differences for the normal population, for groups at risk, and for suspected HFRS patients.

A. Normal population

The existence of virus in a specific region could be determined if about 200 serum samples from normal persons of blood donors were tested against Hantaan, Seoul and Puumala virus antigens by immunofluorescent antibody testing. Immunofluorescent antibodies of Hantaviruses are group specific, and immunfluorescent antibodies from most strains of Hantaviruses cross react with prototype strains of Hantaan virus, Seoul virus and Puumala virus. Such serum specimens could be sent to the WHO Collaborating Centre or designated laboratory for initial study.

B. Groups at risk

Health personnel working in laboratories where HFRS research is being conducted, laboratory workers, animal room

workers, and rodent breeders are groups at risk to Hantaan and related viruses. Sera from animal caretakers should be checked against Hantaan virus at regular intervals to determine the existence of the virus in the animal rooms. It is also neccessary to immediately test sera against Hantaan virus from groups at risk if an outbreak of unknown fever with albuminuria occurs among animal handlers.

C. Suspect HFRS patients

Clinicians should be aware of the existence of HFRS patients in the region. Another good source of serum specimens for a serosurvey of Hantavirus infection is patients who have similar clinical symptoms of leptospirosis, rickettsiosis, dengue, upper respiratory infection, acute abdomen, thrombocytopenia, influenza, non-A/non-B hepatitis, and chronic renal failure because many cases of HFRS are misdiagnosed clinically as such illnesses in HFRS endemic areas (102). Specific serologic testing for the diagnosis of Hantaan or related virus is recommended for patients who show similar clinical symptoms of KHF or NE.

Specific serologic diagnosis of HFRS is made by demonstrating a rise in titer of specific immunofluorescent (IF), ELISA (enzyme-linked immunosorbent assay), and neutralization (N) antibodies against Hantaan and related viruses in sera collected twice during the course of illness at one week interval or longer. IF, ELISA and N antibodies appear during the first veek of symptoms, reach a peak at the end of the second week, and persist for as long as 18 years (27, 70). Specific antibodies to the virus can be detected even in mild and subclinical forms of the disease.

IF antibodies against Hantaan and related viruses can be detected using virus-infected A-549 and Vero-E6 cells. If those are not available, virus-infected *Apodemus mice*, *Clethrionomys mice*, and rat lung sections can be used as substrate. With rodent lung sections, care should be taken that there is no contaminating reovirus or other murine agent. Specific fluorescence of Hantaviruses appears as discrete

pinpoint granules distributed throughout the cytoplasm of the cells. Recently, HI test, radioimmunoassay, immune-adherence passive hemagglutination, and plaque-reduction neutralization tests have been developed, which will facilitate the diagnosis of Hantavirus infection.

2. Epidemiologic sampling

A. Transmission

Three basic transmission cycles account for most human cases of HFRS ; rural, urban, and laboratory animal. In each of these, a rodent or other mammalian host serves as the source of virus, and humans are infected by contact with infectious products of that host. Transmission is generally via the aerosol route, although transmission via rodent bite has been reported. Specific rodent hosts are significant in each of the basic cycles ; however, other potentially significant hosts such as shrews and domestic cats have recently been proposed. In Asia, the rural form is maintained among populations of the striped field mouse, *Apodemus agrarius*, while in Europe, Scandinavia, and Western Russia, the principal host is the bank vole, *Clethrionomys glareolus*. Although human disease has yet to be associated with Prospect Hill virus, this agent seems to be maintained in North America by the vole, *Microtus pennsylvanicus*. In the urban and laboratory animal cycles, domestic rats (*Rattus rattus, Rattus norvegicus*) appear to be the important hosts. The full range of small mammal hosts of Hantaan and related viruses remains to be defined, and no species should be dismissed arbitrarily when conducting epidemiologic studies.

Person to person transmission has not yet been demonstrated for HFRS causing agents, although a viremia does exist during the early stages of illness. The potential exists for transmission though inadvertent self inoculation by hospital staff handling viremic blood. However, extensive experience has not shown this to be a major route of infection.

B. Epidemiologic investigations of human cases

Investigation of human cases of HFRS should begin with an indepth exposure history of the patient to determine the most probable source of infection. Since the incubation period is 5–43 days, the history should concentrate on events over the preceding 3 to 4 weeks. Questions should be posed to identify the presence of small mammals in the patient's home, place of work, or recreational areas. Responses frequently pinpoint the likely source of infection ; i.e., the patient may recall killing mice in the basement, disturbing rodent bedding while cleaning out a barn, or caring for animals at an institution where laboratory rats are maintained.

Once the most likely source has been identified, attempts should be made to capture small mammals in the specific area of suspected exposure. Appropriate live traps are available from several manufacturers (Tomahawk Live Traps, Tomahawk, Wisconsin, USA, for example) for different sized mammals. Care should be taken to ensure that the traps used are the correct size for the hosts to be captured.

Local universities frequently have experts on their staff who may assist investigators with trap selection and placement. Trap baits vary considerably depending on the habitat investigated and the target host's preference.

A mixture of peanut butter and oatmeal is one frequently used bait that attracts many rodent species. The addition of fresh apple slices is especially attractive to voles in some areas. Whatever the bait, care should be taken to place enough in the trap to ensure survival of the captured animal until the traps are next checked, especially in cold weather. If possible, traps should be placed in protected areas away from direct sunlight or freezing cold. If it is possible for captured animals to freeze in the traps, the addition of some grass or other nesting material may help insulate captured animals until traps are retrieved. For most species, traps should be set in the evening and collected the following morning.

When the site of a patient's exposure is known, the habital should be throughly investigated and traps placed at all sites likely to harbor potential hosts, especially rodents. Such sites might include basements, barns, trash heaps, wood piles, garbage dumps, etc. Since infection within host populations might be very focal, care should be taken to ensure that all habitats are explored and that samples taken from each area are fully documented. Captured animals should be processed as soon as possible, and specimens collected should consist of blood samples and lungs (see Chapter 6). Sera and organs should be processed in the field or preserved for later handling in the laboratory following procedures outlined in Chapter 6.
Care should be taken to ensure that an accurate identification (genus and species) is obtained for each animal sampled. If the animal's correct identification is in doubt, the carcass should be preserved following organ removal (frozen or in formalin) and professional assistance sought from a qualified taxonomist.

C. Epidemiologic investigations in the absence of human cases

Often investigations are performed in the absence of recognized human disease. Under such conditions, the investigator must first become familiar with the natural history of the small mammal hosts of interest.
University biology and experimental animal departments and their libraries are good places to start. Once the habits of the animals in question are known, the investigator may sample regions of interest through live trapping as described above. Such studies are sometimes best addressed in two phases : an initial seroepidemiologic survey to identify regions with high numbers of antibody positive rodents, followed by an intense collection effort to obtain material for virus isolation attempts. When large distances are involved or time is limited, dividing the effort may be impractical, and both phases may be accomplished at once.

The critical elements in seroepidemiologic studies are being aware of the frequent, extreme focality of Hantaan and related viruses in small mammal populations and recording in sufficient detail sample collection sites to allow identification of a focus of infection when tabulating the data once all laboratory tests have been completed. Depending on the home-range characteristics of the host, such foci may be quite small. Appropriately large sample sizes should be obtained to ensure that sufficient numbers of individuals are examined to detect foci of infection. Sample size will depend on time available, trap success, and host density. In the absence of carefully designed quantitative protocols, previous studies of urban rat-associated, Hantaan-like viruses have attempted to collect at least 10 individuals of the same species from each discrete habitat sampled.

Once a focus of infection has been identified, frequently a large proportion of that population will be found to contain antigen or antibody to Hantaan or related viruses. Under such circumstances, the probability of successfully isolating virus from samples obtained may be quite good. In addition, if the population is left undisturbed, the virus may be maintained in that habital for a period of years, a potentially significant factor if the goal of the study is disease prevention, avoidance, or control.

While conducting field studies of Hantaan and related viruses, care must to taken to minimize the potential of aerosol transmission. Although many of the safety benefits available to the investigator working in the laboratory will be missing, the field worker should, as a minimum, use masks and gloves and attempt to handle animals in a well ventilated area. See Chapter 9 for additional comments on safety.

D. Arthropod vectors

The question of arthropod vectors of Hantaan and related viruses has received much attention, but little evidence exists to clearly resolve whether this is a significant aspect of the viral maintenance cycle. The absence of arthropods in some

laboratory rat associated outbreaks of HFRS firmly establishes that arthropod vectors are not required for successful transmission ; however, their potential to contribute as secondary or alternate routes of infection remains unresolved. A detailed discussion of arthropod vectors is beyond the scope of this overview and suffice to say that suspect vector species must be epidemiologically relevant to a potential pattern of transmission between infected and susceptible hosts. Blood-sucking arthropods have generally been considered to hold the greatest potential as vectors of Hantaan and related viruses, and successful isolations from mites have recently been claimed. It is difficult, however to be certain that virus recovered from mites does not simply represent ingested viremic blood or tissue exudate. Consequently, if there is question whether arthropods are engorged, care should be taken to ensure that specimens are held at least 48 hours to allow complete digestion of ingested material prior to examination for virus. Otherwise, standard procedures for processing arthropods for virus isolation attempts should be followed.

Chapter VI

Virus Isolation and Identification

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Virus Isolation and Identification

1. Virus isolation

Hantaan and related viruses have been isolated from human clinical samples and small mammal reservoir hosts. Virus recovery has, however, only been accomplished with great difficulty. Isolation techniques will continue to improve as more investigators enter the field and novel approaches are attempted. The methods reported here have been successfully employed, but may well be further refined.

A. Starting material

a. Human material

As described in Chapter 5, Hantaan and Seoul viruses have been recovered from clinically ill patient serum or whole blood drawn during the first 3 to 9 days of illness, with the probability of successful isolation diminishing with the onset of symptoms. Virus isolation has been attempted from human tissues and other body fluids (i.e., saliva, feces, urine) of patients suffering from various forms of HFRS, but no successful isolations from these materials have been reported.

b. Reservoir hosts

Several virus isolations have been made from naturally infected vertebrate hosts of Hantaan and related viruses including :

(1) Prototype Hantaan virus from *Apodemus agrarius coreae* lungs (wild caught animals) and several other organs and excreta (following experimental infection).

(2) Lungs and spleen tissues from *Clethrionomys glareolus* infected with causative agent of nephropathia epidemica.

(3) Lungs and spleen of domestic (*Rattus norvegicus* and

Rattus rattus) or laboratory rats naturally infected with Hantaan and Seoul viruses and urine, saliva, and feces for urban rat, Girard Point, SR-11, Tchoupitoulas strains, and others.

(4) Several other organs and excreta following experimental infection.

(5) Prospect Hill virus from *Microtus pennyslvanicus* lungs.

Lungs appear to be the organ of choice for initial isolation attempts from small mammals.

B. Handling and storage

Preliminary evidence suggests that the rate of successful isolation is greatest when fresh material is used ; however, virus may be recovered from material stored at -70 C, in liquid nitrogen or dry ice. If stored prior to isolation attempts, care should be taken to adequately seal containers to avoid excessive changes in pH and to prevent repeated freeze-thaws.

C. Preparation of samples

Serum or whole blood may be inoculated directly into the substrate used for virus propagation (see Isolation substrates). Tissues should be processed as a 10% or 20% (wt vol) suspension in appropriate diluent (Eagle's mininum essential medium with antibiotics and 10% fetal bovine serum, or equivalent). Best success has been obtained when triturating tissues in a closed mechanical blender (Stomacher Lab Blender, Model 80, Cincinnati, Ohio, USA) ; however, hand mincing of lung or spleen tissues will suffice, especially when cocultivation is attempted in the field. Care should be taken to ensure that no aerosol is generated when mechanical blenders are employed (see Chapter 9, Laboratory Safety). Triturated tissues may be centrifuged at low speed or allowed to stand for several minutes to remove larger tissue fragments, but cells should not be pelleted and resuspended in fresh medium. (We filter the suspension thru 0.4 μ filter before inoculating onto cell cultures). Repeated washing of triturated cells decreases the amount of infectious virus present.

Suspended cells may be inoculated directly into the isolation substrate.

D. Isolation substrates

(1) Experimental animals

Hantaan and related viruses have been successfully isolated in a number of different experimental animals. Specially developed colonies of the natural reservoir hosts (*Apodemus*, *Clethrionomys* mice and others) have been used with varying degrees of success. Laboratory rats are good experimental hosts for most recognized strains of Seoul and related viruses (15). Serologic studies in laboratory outbreaks of HFRS indicate that most inbred and outbred strains of laboratory rats, with the possible exception of OKA and SHR rats, produce a good antibody response when infected. Suckling mice are not good hosts for initial isolation attempts, although they exhibit a generalized infection and death following intracranial inoculation with high-titered, cell culture adapted, prototype Hantaan virus (strain 76-118) and Seoul virus (strain 80-39).

Infection of experimental hosts is detected by demonstration of specific antibody or antigen following inoculation. Intramuscular (0.5 ml) inoculation has produced infection in adult rats ; intrapulmonary inoculation has been attempted with voles and some other hosts. Hantaan virus gives best results when inoculated intrapulmonarily in *Apodemus* mice (3).

The general protocol involves a prebleed, inoculation of the host with triturated tissue suspension, and subsequent bleedings to document development of antibody. In the rat model, specific antibody may be demonstrable by 20 to 30 days postinoculation, but frequently 60 or more days may be required. Once antibody titers reach 512 or greater by immunofluorescent antibody (IFA) assay, antigen is almost always detectable in lungs, and the animal may be sacrificed and lung tissue triturated as described above for attempts to adapt the virus to cell culture.

Passage through an experimental host is not required for successful virus isolation. Hantaan and related viruses have been recovered directly in cell culture. Passage is, however, beneficial when the original material is low titered or contaminated. By using an experimental host initially, the investigator may be assured of the presence of infectious virus in the sample and may have a greater volume of material to use in attempts to adapt the virus to cell culture. Since several blind passes might be needed to adapt the virus to cell culture, the knowledge that the original starting material indeed contained infectious virus may well justify the additional time required for passage through an experimental host.

(2) Cell culture

The cell culture of choice for isolation of Hantaan and related virus is Vero-E6 (American Type Culture Collection, C 1008). Other cell lines that have been successfully employed include A-549 human lung carcinoma cells (ATCC CCL-185) and primary rat lung cells. Isolation in cell culture is accomplished by cocultivation of triturated tissues with Vero-E6 cells or direct inoculations of cell cultures with infectious serum or whole blood. Infection does not produce a cytopathic effect, but rather is detected by the presence of characteristic cytoplasmic fluorescence when examined by IFA technique. Typically 1 to 2 ml serum, blood, or tissue suspension is inoculated onto confluent monolayers of cells grown in 25 cm flasks. Growth medium is added to a final volume of 5 to 7 ml and the flasks incubated at 37° C (Some strains appear to grow better at 35° C).

Following 12-14 or more days incubation, during which the growth medium may or may not have been changed, the medium is decanted, the cell monolayer trypsin disrupted, and the cells suspended in fresh growth medium.
While suspended, several slides are prepared and examined for characteristic Hantaan antigen expression following IFA techniques described below, and 1 to 2 ml suspended cells are cocultivated with freshly planted (50% to 70% confluent)

Vero-E6 cells. Similar passage may be repeated at 7 to 14 day intervals for as many as 50 or more days. Characteristic cytoplasmic fluorescence is usually seen during the first 10 to 20 days in culture, but often requires much longer. Generally, a small proportion of the cells will exhibit antigen initially ; but with repeated expansion, virtually 100% become infected. Prior to reaching that stage, however, the proportion of cells expressing antigen may rise and fall from less than 10% to about 50%, then back to 10%.

In the early stages of passage, virus is usually of such low titer in the culture fluids that it can not be detected on either plaque assay or passage to uninfected cells. Once the majority of cells are antigen positive, however, infectious virus is usually detectable in culture fluids and passage from cell-free media is possible. Repeated passage increases the virus titer, but the optimal interval between passages varies considerably among virus strains, and a growth curve is usually required to maximize infectious virus production.

2. Virus identification

A. Immunofluorescent antibody technique (IFAT)

If the techniques described above are used to isolate the virus, a presumptive identification will already have been made through the detection of antigen, which reacted with Hantaan-specific antibody in the IFA technique. The IFA technique is broadly reactive among the several Hantaan-related viruses and is, thus, an excellent screening tool (see Chapter 8). Specific identification can also be made using this technique if monoclonal antibodies are employed however, differences between Hantaan and related agents are often most apparent when other, more specific assays are employed.

B. Plaque-reduction neutralization tests (PRNT)

The plaque-reduction neutralization test (see Chapter 8) is quite specific and will readily distinguish between prototype Hantaan and other closely related viruses. To date, about a

tenfold or greater difference has been detected between homologous and heterologous reactions when prototype Hantaan was compared with viruses isolated from domestic rats. Differences among several viruses isolated from domestic rats have generally not been as great, yet this test appears to hold promise for their differentiation as well.

C. Other techniques

Most of the remaining tests discussed in Chapter 8 can be used in identifying Hantaan and related viruses. Insufficient information is presently available to indicate which tests will be of greatest value. Certainly the enzyme immunoassay will receive greater attention in the future due to its ease of operation and lack of supporting equipment needed. Separation of very closely related strains requires more sophisticated techniques such as oligonucleotide fingerprinting, which are beyond the scope of this manual.

D. Contaminating agents

Investigators should be aware that contaminating agents such as reovirus, mycoplasma, bacteria, and fungi may be coisolated with Hantaan and related viruses. One indication of the presence of a contaminating agent may be cytopathic effect in cell culture, since all known Hantaan and related viruses do not destroy cells. Care should be taken to rule out the presence of contaminating agents prior to distribution of isolated viruses. This may pose a significant problem, especially in the cases of reoviruses and mycoplasma, since they are ubiquitous organisms, and both their detection and control may be difficult. Most types of reovirus can be detected by IFA using polyvalent antireovirus immune serum. Removal of reovirus from Hantaan virus isolates has been accomplished with great difficulty by repeated passage in cell culture in the presence of reovirus-type specific immune sera.

Chapter VII

Electron Microscopy of Hantaviruses

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Electron Microscopy of Hantaviruses

1. Introduction

Hemorrhagic fever with renal syndrome (HFRS) is a collection of related diseases causing world-wide epidemics. One of the etiologic agents, Hantaan virus, was first isolated by Ho Wang Lee and his coworkers in 1976 (1, 2).
The morphology of this virus, however, was not revealed until 1982 and 1983 when McCormick et al (47), White et al (48), and Hung Tao et al (49, 103) first described virus morphology observed by negative staining and thin-section electron microscopy, respectively.

There are numerous electron microscopy (EM) methods available and each has advantages that allow one to choose from them according to the intended purpose. For example, thin-section EM can be used to study virus structure and virus-cell relationship by providing the opportunity to localize viral antigens within cells by antibody labeling. Thin-section procedures are tedious in comparison to negative-staining EM, which is easier, more rapid, and provides better resolution of virion surface structure. Negative staining, however, requires relatively high virus concentrations. The suspension to be examined must contain 10^6-10^9 particles per ml.

The following procedures have been successfully applied to the EM visualization of Hantaviruses.

2. Procedures of EM techniques

A. Thin-section EM

(1) Susceptible monolayer cell cultures infected with Hantaviruses are usually harvested 10-15 days postinoculation, at which time about 90% of the

cells are infected as revealed by *immunofluorescence.*

(2) Cells are scraped from bottles with a rubber policeman and transfer them to a centrifuge tube.
(3) Centrifuge at low speed (3,000 rpm) for 20 minutes to pellet cells, discard the supernatant.
(4) Fixation with 2.5% buffered glutaraldehyde for 30-60 minutes at 4 C, constitutes the *prefixation* procedures.
(5) Wash with Palade's buffered solution for 30 minutes.
(6) Fix again with 1% buffered O_sO_4 for 30-60 minutes, as a *postfixation.*
(7) Wash with Palade's buffer solution for 30 minutes.
(8) Wash with graded ethanol, starting from 50% and proceeding to 70%, 90%, and 100%, each for 20 minutes, to accomplish *dehydration.*
(9) Infuse with epoxy resin Epon 812 at 37 C overnight.
(10) Embed in Epon 812 and polymerize at 60 C for 48 hrs.
(11) Cut with glass or diamond knives to make thin sections.
(12) Thin sections are double stained with 2% uranyl acetate and lead citrate, 30 minutes each.
(13) Thin sections prepared according to the procedures detailed above are ready for EM examination.

B. Thin-section enzyme (horseradish peroxidase) EM

This is an immunological procedure in which enzyme conjugated antibodies react with viral antigens ; and as a result of the antibody-antigen reaction, the enzyme forms an electron dense precipitate following substrate addition.

It is, in fact, a combination of cytochemistry and thin-section EM. The essential steps in the procedure are :

(1) Cell cultures infected with Hantaviruses are prefixed with periodate-lysine-paraformaldehyde (PLP) for 1 3 hrs at 0 C. PLP is prepared by adjusting the pH of a 0.15 M solution of lysine HCL in distilled water to 7.4 with 0.1 M sodium phosphate buffer, pH 7.4 to twice the volume of lysine HCL solution. Just before use, one part

of an 8% paraformaldehyde solution is combined with three parts of the lysine-phosphate buffer and solid sodium m-periodate is added to a final concentration of 0.01 M.

(2) Cover the monolayer culture with convalescent serum (hyperimmune or monoclonal antibodies) and incubate at 37° C for 30 minutes.
(3) Shift to 4° C for 6 hrs or overnight.
(4) Wash 5 times with buffered saline at room temperature with gentle rotation.
(5) Label with enzyme (horseradish peroxidase) conjugated protein A and incubate at 37° C for 30 minutes.
(6) Fix with 2.5% buffered glutaraldehyde for 30 minutes at room temperature.
(7) After extensive washing with PBS, the cells are incubated with a 0.05% solution of diaminobenzidine (DAB) in 0.05 M Tris-HCL buffer, pH 7.6 for 30 minutes with continuous shaking, followed by an incubation with 0.02% DAB and 0.05% H_2O_2 in the same buffer.
(8) Postfixation with O_sO_4. The cells are washed extensively and treated with O_sO_4 in PBS for 1 hr.
(9) Dehydration and embedding. After thorough washing, the cells are dehydrated with graded acetone or ethanol and embedded in Epon 812.

C. Negative stain EM

Procedures for conventional negative staining :

(1) Cover a 400-mesh copper grid with 0.5% formvar and a thin layer of carbon.
(2) Secure grid in forceps and suspend over petri dish.
(3) Make a suspension of specimen with 1% ammonium acetate.
(4) Place a drop of liquid on suspended grid. Draw off excess by blotting the side of the liquid droplet with filter paper.
(5) Place a drop of 2% phosphotungstate (pH 6.4 to 7.2) on grid. Wait approximately 1 minute.

(6) Use filter paper to remove excess stain. Release grid to the dish.
(7) Expose approximately 6 inches from UV light for 3 to 5 minutes (the stain itself, does not inactivate viruses).
(8) View with the aid of a transmission EM at 20,000 X.

Preparation of cell culture propagated or purified virus for Immune Electron Microscopy (IEM) :

(1) Centrifuge serum at 15,000 rpm for 1 hr and dilute serum with PBS in fourfold serial dilutions.
(2) Combine 0.9 ml serum + 0.1 ml undiluted virus, and mix well.
(3) Incubate at 4 C overnight.
(4) Centrifuge 90 minutes at 18,000 rpm.
(5) Resuspend pellet in one drop distilled water · 3% PTA.

Pseudoreplica technique for negative staining of viral specimens for EM (This method employs concentrated virus and frequently yields better results). Method adapted from Palmer (104) :

(1) Place specimen on block of 2% agarose and allow to dry.
(2) Drop 0.5% formvar solution on surface and quickly drain excess onto absorbent paper.
(3) Trim edges of agarose with scalpel so that formvar film has sharp edges ; float formvar film, with virus attached, onto surface of stain.
(4) Place EM grid on formvar film.
(5) Pick up grid and film with stainless steel post, turning formvar-virus surface up.
(6) EM examination.

D. Brief description of the morphology of Hantavirus (Fig. 10, 11)

Size and shape of the virions :

(1) The virus particles of hantaviruses are spherical or oval in shpae.
(2) The mean diameter is 122 nm, may vary from 78 to 210 nm.

(3) A polymorphic appearance is characteristic of this group of viruses.

Virion structure :

(1) A distinct envelope (membrane) with numerous short, fuzzy projections surround the virions. Projections appear as a grid-like lattice in negative stained preparations.

(2) Enclosed inside the envelope are granulo-filamentous nucleocapsids, which are larger than those observed with other members of Bunyaviridae.

Cytoplasmic inclusion bodies of Hantavirus :

Three types of inclusion bodies have been described :

(1) Granular inclusion bodies that are spherical and consist of numerous tiny granules.

(2) Filamentous inclusion bodies appearing as long bundles and often irregular in shape.

(3) Granulo-filamentous inclusion bodies that are a combination of the above types. These may be very large, approaching the size of the cell nucleus (105).

Method of preparation of Palade's fixative (Palade 1952).

1. Prepare a veronal-acetate stock solution with :

sodium veronal (barbitone sodium)	2.89 g
sodium acetate (anhydrous) or	1.15 g
sodium acetate (hydrated)	1.90 g
distilled water to make	100 ml

This solution is stable and keeps for some months at 4° C.

2. Prepare the veronal-acetate buffered fixative with :

2% osmium tetroxide in water	12.5 ml
veronal-acetate stock solution	5.0 ml
0.1 N HCL	5.0 ml (approx.)
distilled water to make	25.0 ml

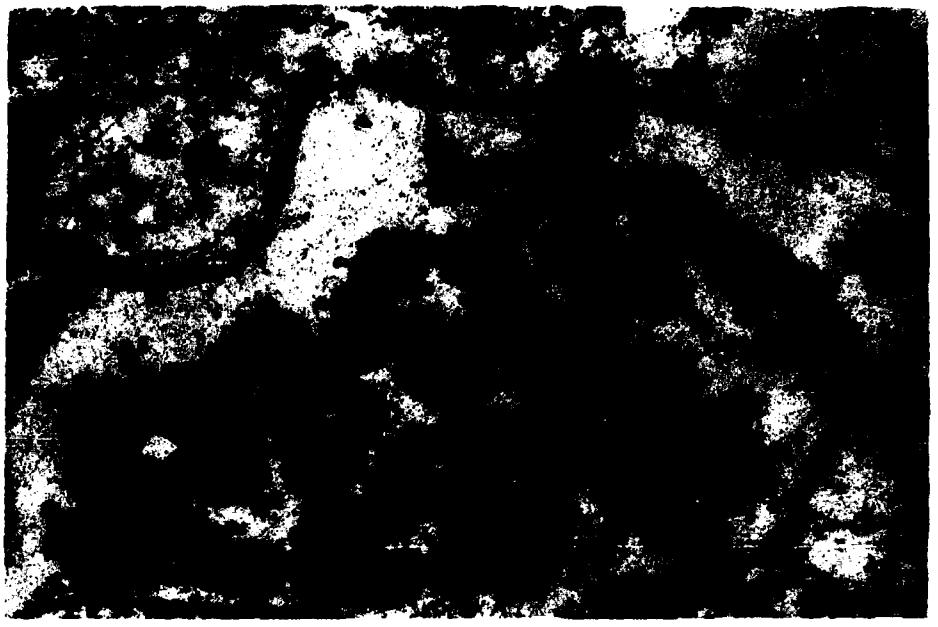

Fig. 10. Thin-section EM of Seoul virus (R22) showing a cluster of virions released into the intercellular space, the ribosome-like dense granules are virion-associated granules.

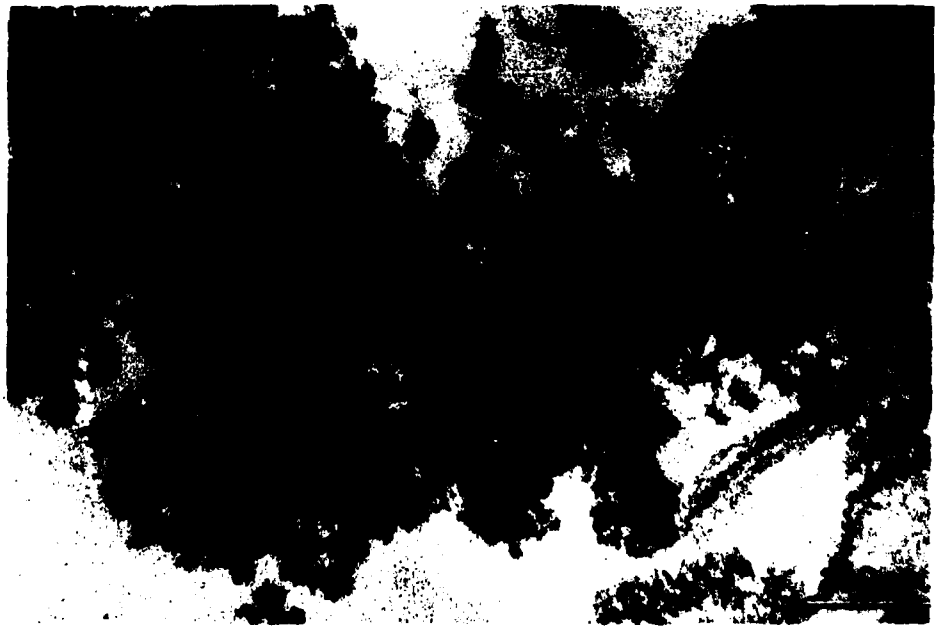

Fig. 11. Thin-section EM showing Seoul virus labeled with antibody-conjugated horseradish peroxidase elctron-dense precipitates of enzyme represent specific immune reaction.

Chapter VIII

Serologic Techniques for Detection of Hantaan Virus Infection, Related Antigens and Antibodies

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1. Immunfluorescent antibody technique

Immunofluorescent antibody technique (IFAT) was successfully employed by Lee and Lee (1) in 1976 to detect Hantaan virus antigen from lungs of *Apodemus agrarius* mice and to demonstrate antibody against the antigen in sera from patients with KHF and in the sera from wild rodents. This technique has been widely applied in virologic, sero-epidemiologic, and pathogenesis studies of Hantaan and related viruses (3, 30, 46).

Even though several techniques have recently become available to detect the antibodies and antigens of Hantaan and related viruses, IFA technique remains the most reliable, group specific and sensitive method for the study of HFRS.

A. Antigen preparation and fixation

a. To prepare frozen sections of virus-infected and uninfected tissues :

(1) Mount tissue (measuring approximately 5 $mm^2 \times 3$ mm) on a cryostat chuck using a drop of embedding medium (OCT compound, Tissue-Tek Ⅱ) and freeze at -25° C.

(2) Transfer 4 to 6 micron thick, cryostat-cut sections onto slides and allow sections to dry at room temperature.

(3) Store slides at -70° C.

Comments : (i) The sections should be attached to the slide without overlapping or creasing, which would intensify nonspecific fluorescence. (ii) The prepared section should be fixed and stained immediately. If necessary, the section can be stored at -70° C before or after fixation.

b. For virus-infected and uninfected cell smears :

(1) Trypsinize virus-infected and uninfected cell monolayers and suspend cells in Eagle's minimal essential medium

containing 5% heat inactivated fetal calf serum.

(2) Centrifuge cell suspensions at 1,000 rpm for 5 min, and resuspend cell pellets in twice the volume of medium, mix well, and spot slides. Allow slides to dry at room temperature.

(3) Store slides at -70 C.

Comments : This method of preparing cell virus antigen is easy and simple, but care should be taken to prepare as thin and well-spread a cell layer as possible.

c. Intracellular virus antigen detection :

(1) Fix tissue sections or cell spots with either 100% acetone or a mixture of periodate lysine paraformaldehyde (PLP) for 7 to 10 min. at room temperature.

(2) Wash slides in 0.01 M phosphate buffered saline (PSB) for 5 min. and rinse briefly with distilled water. Allow slides to dry completely before proceeding with staining.

Comments : Excellent preparations of cell spots have been obtained by fixation with PLP mixture. Furthermore, antigens in cytoplasm, as well as in cell morphology, have been clearly demonstrated. In contrast, preparations with poor contrast and dull cell shapes have been obtained by fixation with acetone.

B. Direct IFA technique

a. To detect viral antigens in animal tissues or in cultured cells :

(1) Prepare fluorescein isothiocyanate (FITC) labeled IgG to Hantaan or related virus. Add 8 units FITC conjugate on fixed tissue sections or cell spots thought to contain viral antigen.

(2) Incubate slides for 30 min. at 37 C in a moist chamber. Wash slides twice with 0.01 M PBS. Briefly rinse slides with distilled water. Place a drop of buffered glycerin and coverslips on slides. Examine slides using a fluorescence microscope.

b. To measure antiviral (blocking) antibodies :

(1) Prepare serial dilutions of serum samples in 0.01 M PBS. Place 10 to 15 μl of each serum dilution on fixed, virus-infected tissue sections or cell spots.
(2) Incubate slides overnight at 4° C in a moist chamber. Wash slides twice with 0.01 M PBS.
(3) Dry slides completely, then add 8 units FITC-labeled IgG prepared against Hantaan virus.
(4) Incubate slides for 30 min. at 37° C in a moist chamber. Wash slides twice with 0.01 M PBS. Briefly rinse slides with distilled water. Place a drop of buffered glyccrin and coverslips on slides. Examine slides using a fluorescence microscope. Compare the intensity of fluorescence with that obtained with known positive and negative serum samples. Express the blocking antibody titer as the reciprocal of the highest serum dilution at which a reduction in fluorescence results.

Comments : (i) This is the simplest and most reliable method of measuring viral antigen, but it is less sensitive than the indirect method. (ii) However, with this method, each antiserum should be labeled with the fluorochrome. This method is preferred for proving specificity and determining blocking antibody rather than for measuring viral antigen.

C. Indirect IFA technique

a. To detect viral antigens in animal tissues (Fig. 12) or in cultured cells (Fig. 13) :

(1) Add 16 fluorescent antibody units of a known positive serum and the same dilution of known negative serum to fixed tissue sections or cell spots thought to contain viral antigen.
(2) Incubate slides for 30 min. at 37° C in a moist chamber. Wash slides twice with 0.01 M PBS.
(3) Dry slides completely, then add 8 units FITC-labeled

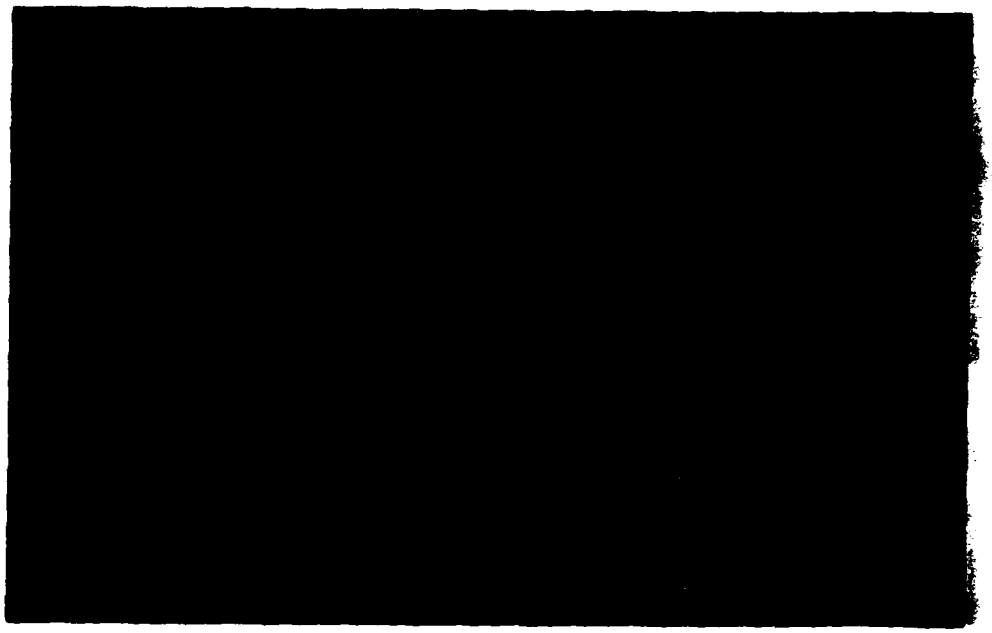

Fig. 12. Specific fluorescence (fluorescing spots) observed in lung section from *Apodemus agrarius coreae* infected with Hantaan virus by the indirect immunofluorescent antibody (IFA) test. Magnification ×600.

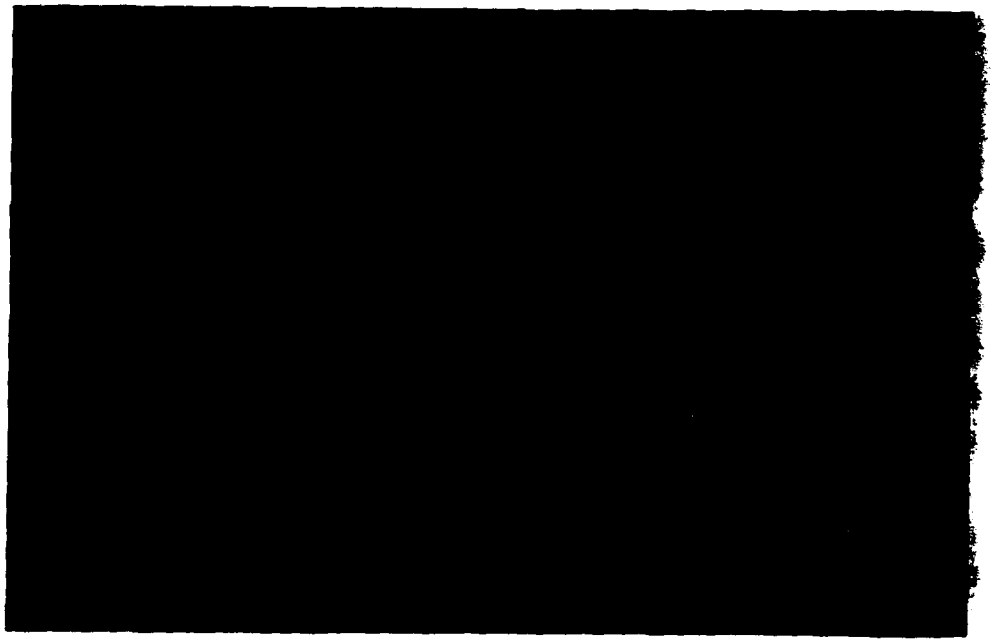

Fig. 13. Specific fluorescence of Hantaan virus antigen in Vero-E6 cells stained by the indirect immunofluorescent antibody test. The discrete intracytoplasmic fine granules are viral antigens on 6 days after inoculation. Magnification ×900.

antibody prepared against the primary antibody containing Evan's blue (1 : 150,000).

(4) Incubate slides for 30 min. at 37° C in a moist chamber. Wash slides twice with 0.01 M PBS. Briefly rinse slides with distilled water. Place a drop of buffered glycerin and coverslips on slides. Examine slides using a fluorescence microscope.

b. To measure antiviral antibodies :

(1) Prepare serial dilutions of serum samples in 0.01 M PBS. Place 10 to 15 μl of each serum dilution on fixed virus-infected tissue sections or cell spots.

(2) Incubate slides for 30 min. at 37° C in a moist chamber. Wash slides twice with 0.01 M PBS.

(3) Dry slides completely, then add 8 units FITC-labeled antibody prepared against the primary antibody.

(4) Incubate slides for 30 min. at 37° C in a moist chamber. Wash slides twice with 0.01 M PBS. Briefly rinse with distilled water. Place a drop of buffered glycerin and coverslips on slides. Examine slides using a fluorescence microscope. Express the fluorescent antibody titer as the recriprocal of the highest serum dilution at which specific fluorescence is observed.

Comments : (i) This method is more sensitive than the direct method. However, as the reacting reagent is doubled, specificity becomes less reliable and more time is required for staining. (ii) The known positive sera should be titrated against known antigen to determine optimal working dilutions. The highest dilution at which specific fluorescence is observed is regarded as 1 unit, then 16 times lower dilution from the dilutions used for working dilution (16 units).
(iii) To determine units of antibody. The 8 units at a working dilution is determined, as above. (iv) The color for specific staining is a brilliant greenish-yellow on a red background, which is stained by Evan's blue.

D. Reagents for IFA techniques

Phosphate-buffered saline (0.01 M, pH 7.4) requires :

$NaH_2PO_4.H_2O$	0.40 g
$Na_2HPO_4.12H_2O$	2.54 g
NaCl	8.50 g

Dissolve reagents in 1,000 ml of distilled water.

A PLP mixture, using phosphate-buffered saline as the solvent, is made with the following concentration of reagents :

0.1 M Lysine-monohyrate-HCl
2% paraformaldehyde
0.01 M sodium m-periodate

Before it is brought to a final volume, the pH is adjusted to 7.4.

A buffered glycerin solution requires :

0.01 M PBS (pH 7.4)	1 vol.
Glycerin (free of autofluorescence)	9 vol.

The two reagents are mixed throughly.

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2. Enzyme immunoassays

The rapid and accurate diagnosis of hemorrhagic fever with renal syndrome is critical to the good care and management of infected patients. Until recently, however, no single test was sufficiently sensitive and specific to accurately diagnose HFRS in patient seen early in their disease. The IgM capture enzyme immunoassay described in this section holds promise to fill that void. The test has been extensively field tested in both Korea and China using clinical specimens drawn from patients at or soon after their admission to hospitals. To date, sera from several hundred Asian patients with a clinical diagnosis of HFRS have been examined using these tests. In general, greater than 95% of those Hantaan virus-infected patients had demonstrable IgM antibody in the first or second serum sample tested following hospitalization. Thus, the test appears to be quite sensitive. Sera from several hundred healthy individuals have been examined, and a false positive rate for IgM antibody of approximately 1% has been found. Lesser numbers of sera from acutely ill patients infected in Greece and Yugoslavia with severe HFRS have been examined using the IgM capture test, with results similar to those found with Asian patients. Limited testing on sera from patients infected with Puumala virus, cause of nephropathia epidemica in Scandinavia, also suggest that their sera will react in the IgM capture test, but to somewhat lower titers than if Puumala viral antigen is used.

Very high IgM titers (i.e., greater than 1 : 5,000) are frequently seen in the initial acute serum sample tested. Elevated IgM antibody titers persist for several weeks, then decline by 3 to 6 months, although low levels of specific IgM antibody can persist for 1 year or more.

IgG antibody appears with or slightly later than IgM antibody, at lower titers initially, but rapidly increases in titer and persists for many years. Thus, the IgM test, in combination with the IgG test, is a powerful diagnostic tool.

The ability of enzyme immunoassays to readily detect virus-specific immunoglobulin G and M antibodies is well established (107, 108). In the tests developed for measurement of hantaviral antibodies described below, several of the steps were common in both the IgG and IgM assays. Polyvinyl plates (Dynatech Laboratories, Alexandria, VA, USA) were used as the solid phase, and the volume for each step was 100 μl in all cases. The wash solution was phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Tween-20 detergent, and all dilutions were in PBS with 0.1% Tween-20 and 5.0% fetal bovine serum. Unless otherwise noted, all incubations were at 37° C for 45-60 minutes, the substrate for the peroxidase conjugated antibodies was 2-2'-azino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), which produces a blue-green color when positive that can be read in a spectrophotometer at wavelength 414. Optimum dilutions of each hyperimmune antibody reagent were determined by checkerboard titrations. The positive antigen was Hantaan virus strain 76-118 grown in Vero-E6 cells, concentrated, inactivated and safety tested. The negative antigen was similarly handled in Vero E-6 cell supernatant.

A. IgG ELISA

Because of the difficulty of safely growing and purifying Hantaan virus for direct coating of antigen to the polyvinyl plate, an indirect method of coating the antigen was utilized. A mixture of mouse ascitic fluid containing five monoclonal antibodies, which included antibodies directed against both nucleocapsid and glycoproteins of Hantaan virus, was diluted 1 : 5,000 in PBS and coated to the plate for 14 hours at 4° C. After washing, Hantaan antigen (or negative control,

cell culture antigen) was diluted 1 : 40 and added to every other row (alternating positive and negative control antigens) of wells in the plate. After incubation and washing, two-fold dilutions of sera were made starting at 1 : 100, and each dilution was added to at least 4 wells. After incubation at 37° C for 1 hour, then washing, peroxidase conjugated affinity purified mouse anti-human IgG (gamma chain specific) antibody (Accurate Chemical and Scientific Corp., Westbury, NY, USA) was diluted 1 : 16,000 and added to each well. Incubation and washing were followed by the addition of substrate and reading of the optical density (OD) after 60 minutes at 37° C.

B. IgM ELISA

Because of potential competition by immune IgG molecules and possible false positive reactions caused by rheumatoid factor, we used an IgM trapping format, which is not influenced by these variables. Goat hyperimmune, affinity-purified anti-human IgM (mu chain specific) (Tago Inc., Burlingame, CA, USA) was diluted 1 : 500 in PBS and coated to the polyvinyl plate by incubation at 4° C for 14 hours. After washing, sera diluted two-fold starting at 1 : 100 were added to at least 4 wells and incubated to 60 minutes at 37° C. After washing, anti-Hantaan specific IgM molecules trapped to the plate were detected by the addition of a 1 : 10 dilution of positive antigen (or negative control antigen). After incubation, then washing, rabbit anti-Hantaan diluted 1 : 2,000 was added to all wells. After incubation and washing, peroxidase-conjugated anti-rabbit antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) diluted 1 : 2,000 was added. The plates were incubated, then washed, and substrate was added and the OD was read after 60 minutes incubation at 37° C.

Interpretation of results:

A specific activity OD value was determined for each dilution by subtracting the nonspecific background OD in the

well with the negative antigen from the OD recorded in the well with the positive antigen. A positive specific activity was greater than or equal to 3 standard deviations from the mean value of corresponding dilution of a series of known seronegative controls.

C. Formulations

(1) Coating buffer (PBS) :

Sodium chloride (NaCl)	8.0 g
Potassium chloride (KCl)	0.2 g
Potassium phosphate (monobasic-KH_2PO_4)	0.2 g
Sodium phosphate (dibasic-$Na_2HPO_4 \cdot 12H_2O$)	2.9 g

Make up to 1 liter with distilled water. Sodium azide (NaN_3) at 0.2 g/l can be added to prevent microbial growth, but peroxidase is inhibited by azide ; use 0.01% thimerosol when using peroxidase conjugates. Store at 4° C. pH should be 7.4.

(2) Washing buffer (PBS-Tween):

Sodium chloride (NaCl)	8.0 g
Potassium chloride (KCl)	0.2 g
Potassium phosphate (monobasic-KH_2PO_4)	0.2 g
Sodium phosphate (dibasic-$NA_2HPO_4 \cdot 12H_2O$)	2.9 g
Tween 20	0.5 ml

Make up to 1 liter with distilled water. Sodium azide (NaN_3) at 0.2 g/l can be added to prevent microbial growth, but peroxidase is inhibited by azide ; use 0.01% thimerosol when using peroxidase conjugates. Store at 4° C. pH should be 7.4.

(3) Diluting buffer (PBS-T + 5% FBS or 1% BSA) :

Add bovine serum albumin to the PBS-T washing buffer to a final concentration of 1.0% or add 5% FBS. Also add 500 μg/ml dextran sulfate (optional). Add BSA or serum to diluent buffer the day of the test ; do not store more than 24 hours.

(4) Substrate buffer (diethanolamine buffer) for alkaline phosphatase enzyme conjugates :

Diethanolamine	97 ml

Distilled H_2O	800 ml
Sodium azide (NaN_3)	0.2 g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	100.0 mg

Add 1M HCl to above until the pH reaches 9.8, then add distilled water to a final volume of 1 liter. Store at 4 C in the dark.

Substrate buffer for peroxidase conjugates varies depending on which of the many substrates one uses (see references). However, we recommend using ABTS, which can be purchased to final dilution (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA).

(5) Stopping solution for alkaline phosphatase :
Sodium hydroxide (NaOH) – 120.0 g
Add distilled water to 1 liter.
Stopping solution for peroxidase varies depending on substrate.

D. Materials required

a. Reagents :

(1) Hantaan virus antigen (inactivated) and a negative (control) antigen ; Hantaan antiserum (monoclonal mix and polyclonal rabbit).
(2) Horseradish peroxidase or alkaline phosphatase conjugated antirabbit immunoglobulin.
(3) Buffers for coating antigens to plate, washing, diluting samples, and diluting substrate.
(4) Fetal bovine serum.
(5) Substrate : 2, 2-azino-di-(3-ethyl-benzthiazoline-sulfonate), abbreviated ABTS (for peroxidase) or p-nitrophenyl phosphatase (for alkaline phosphatase).

b. Equipment :

(1) Microtiter plates.
(2) Incubator (37° C) and refrigerator (4 C)(recommended but not essential).
(3) Spectrophotometer (recommended but not essential).
(4) Pipettes (0.1 ml, 5.0 ml, 10.0 ml).
(5) Glassware (flasks, test tubes).

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3. Immunosorbent methods for the detection of HFRS antigens and antibodies

The laboratory of Arenaviral Infections (Institute of Poliomyelitis and Viral Encephalitides, AMS of the USSR, Moscow) has developed the optimal conditions for applying solid-phase radioimmuno assay (SPRIA) and ELISA to the detection of specific Hantavirus antigens and appropriate antibodies

Some immunologic aspects and particulars of the epidemiology as well as epizoology in various natural foci of HFRS in the USSR were conducted using immunosorbent assays (SPRIA and ELISA).

A. General characterization of ELISA and SPRIA tests

The highly adsorptive properties of some plastics (solid-phase) can firmly bind different proteins, in particular immunoglobulins (antibodies). Adsorption of specific anti-Hantavirus IgG is performed in the wells of polystyrene or polyvinyl microtiter plates (sensitization of solid-phase). The remaining active binding sites of the solid-phase are blocked with an abundance of nonspecific protein, for example bovine serum albumin (BSA). The Hantavirus test antigen is added next. It reacts with anti-Hantavirus IgG in the bottom of the plate. At the end of each step, the wells are emptied and washed five or six times. Following the washes, anti-Hantavirus IgG conjugated with horseradish peroxidase (HRP IgG) for ELISA or anti-Hantavirus IgG conjugated with ^{125}I(^{125}I-IgG) for SPRIA is added to solid phase, anti Hantavirus IgG-Hantavirus antigen complex.
The quantity of conjugate linked to the solid phase is in proportion to the quantity of bound antigen. The unbound

conjugate is removed and the wells are washed. The quantity of bound conjugate is determined by adding a substrate indicating solution (ortho phenylendiamine peroxide), which turns yellow in the presence of peroxidase. The result can be measured with the naked eye (approximate) or a spectrophotometer.

When SPRIA is used, the quantity of conjugate bound with the solid-phase is estimated in a gamma counter. Sera are screened for HFRS antibodies in a blocking test with 8 units of HFRS antigen according to the scheme : solid-phase antibody-Hantavirus antigen (8 units)-test serum-conjugate (HRP-IgG or ^{125}I-IgG). If the test serum contains antibodies to a Hantavirus, the intensity of the color of the substrate indicating solution is diminished using ELISA and the bound radioactivity is diminished with SPRIA. Normal antigens, sera, and immunoglobulins of the same origin are used as controls.

Immunosorbent methods have the following advantages compared with IFA techniques :

(1) Increased sensitivity, high reproducibility, and accuracy (sera are tested with 8 units of antigen irrespective of antigen activity).
(2) Objective evaluation of results.
(3) Opportunity for direct detection of antigen and antibodies in any materials (suspensions of tissues, ectoparasites, blood, urine, liquor, exudate, and tissue culture fluids, etc.).
(4) High specificity (nonspecific reactions have not been observed even when 20% to 40% crude suspensions of tissues or native sera are tested).
(5) Possibility for quantitative evaluation of antigens.

In contrast with IFA techniques, immunosorbent methods can detect antibodies in sera within a week of disease onset. Between the third and fourth weeks following disease onset, the sensitivity of these methods is equal.

B. Direct "double-sandwich" ELISA for Hantavirus antigen detection and titration

5-10% lung tissues and other organ suspensions, excretions, and ectoparasites of rodents trapped in natural foci of HFRS, as well as human tissue suspensions from fatal cases of HFRS, can be tested for Hantavirus antigen. Blood of rodents or HFRS patient and tissue culture fluids of Hantavirus or HFRS patient and tisue culture fluids of Hantavirus infected cells may be used as antigen. Controls are materials of the same origin as the tested samples, but without HFRS antigen. For screeing, the test and control specimens are examined undiluted and in a twofold dilution series.

Next, 75 ml antivirus IgG isolated from HFRS convalescent sera, with antibody titers no less than 1 : 16,000 (ELISA or SPRIA) containing 10 to 50 mg/ml protein in 0.1 M PBS, pH 7.4, is placed in each microtiter well. For the control, 75 μl normal human IgG solution (without anti-HFRS virus antibodies) containing the same amount of protein is added. Plates are covered and incubated 37 C for 3 hrs (or at 4 C overnight). The unadsorbed globulin is removed by aspiration and the wells washed three times, 5 min. each, at room temperature with 1% BSA in 0.1 M PBS. pH 7.4.

After washing and drying, 50 μl virus antigen diluted in PBS is added. Control antigen (suspension of tissues from noninfected rodents) is added in the same manner. Each test for the detection of HFRS antigen must be accompanied by a control-reference HFRS antigen of known titer. The plate is covered and incubated for 18 hrs at 4 C (or 1.5 hr at 37 C). The unadsorbed viral antigen is removed, and the wells are washed four or five times with 0.05% Tween-20 in PBS and dried. Then 50 μl IgG-peroxidase conjugate in a working dilution is added to each well. The conjugate is obtained by gel filtration of HFRS convalescent serum and conjugation of IgG with horseradish peroxidase (Type VI, RZ 3) performed by the method of Nakane and Kawaoi (109) as modified by

Mathiesen, et. al (110). The plate is again covered and incubated for 1 hr at 37 C. The unadsorbed conjugate is removed, the wells are washed four or five times and dried.

A freshly prepared solution of ortho phenlylenediamine (0.4 mg/ml) with 0.006% peroxidase in citrate buffer (pH 5.0), made by mixing 98.6 parts of 0.1 M citric acid and 101.4 parts of 0.2 M Na_2PHO_4, is used as the substrate and must be used within 5 min. of preparation. A 100 μl aliquot of the substrate is added to each well. After 50 min. of incubation in the dark at room temperature, the reaction is stopped by the addition of 2 M H_2SO_4. In the presence of HFRS antigen, the well's contents turn yellow. The result is measured in a spectrophotometer at 492 nm and considered positive if the ratio of sample to control (P N) is $\geqq$2.1.

C. Direct "double-sandwich" block ELISA for HFRS antibody detection

This method is a modification of those previously described. After specific globulin and BSA adsorption, 50 μl HFRS antigen is added at a dilution four to eight times lower than the endpoint antigen titer determined in previous tests (4 to 8 units). The antigen is incubated as mentioned above, the unadsorbed antigen is removed, and the wells are washed and dried. Then 50 μl test sera are added at dilutions of 1 : 8 and higher. Negative controls are normal human serum without HFRS antibodies and normal control antigen of the same origin as antigen positive material. Positive control is a reference HFRS serum with known titer.

After incubation for 1 hr at 37 C or 18 hrs at 4 C, sera are removed, the wells washed and dried. Then the peroxidase conjugate at a working dilution is added.
The remainder of the test is analogous to that described for the "double sandwich" ELISA. Results are interpreted in the following way. In the wells with normal control antigen, no color must be in evidence. The standard antigen is considered satisfactory if the ratio of positive wells to normal serum or normal antigen (P N) is $\geqq$2.1. Then the color

of wells containing test sera is measured. If the test sera contain specific antibodies, they block the antigen, and the reaction well shows no yellow color. The serum titer is expressed as the highest dilution of test serum blocking more than 50% of the antigen, as compared to controls.

D. Direct "double-sandwich" SPRIA for HFRS antigen detection and titration

The test principles for antigen detection and titration are essentially the same as for ELISA. Specific anti-HFRS IgG is obtained in the same way. Hunter and Greenwood (111) as modified by Purcell, et al (112).

Wells of soft polyvinyl-chloride plates are precoated with 75 μl of anti-HFRS IgG containing 10 to 50 mg/ml protein in 0.1 M PBS solution (pH 7.4). The IgG source is convalescent serum with a specific antibody titer of more than 1 : 16,000 (SPRIA). Control wells are precoated with 75 μl normal human IgG at the same concentration. After 3 hrs incubation at 37° C (or overnight at 4° C), the unadsorbed IgG is removed.

Wells are washed three times with 2% BSA in PBS or 10% bovine serum (BS) in PBS. Plates are dried and 50 μl aliquots test antigen and normal antigen are added. Every test for HFRS antigen detection is accompanied by a specific reference HFRS antigen control with known SPRIA titer. The plate is covered and incubated for 18 hrs at 4° C or 1.5 hr at 37° C. The unadsorbed antigen is removed, and the wells are washed four or five times for 5 min, each and then dried. To each well, 50 μl radioactive conjugate (^{125}I−anti-HFRS IgG) is added. The conjugate is diluted with 1% BSA or 10% BS in PBS so that the radioactivity in 50 μl solution is 100,000 counts/min. approximatly. The plate is covered and incubated 1 to 1.5 hr at 37° C. The unadsorbed conjugate is removed, and the wells are washed carefully five times with 10% BS solution in PBS. The plate is dried, the wells are cut with scissors, and the radioactivity of individual wells measured in a gamma

counter. The antigen titer is the highest dilution of sample in which the radioactivity is at least 2.1 times the highest negative control specimen radioactivity.

E. Direct "double-sandwich" block SPRIA for HFRS antibody detection

This method is a further modification of the tests previously outlined. After specific globulin adsorption is the bottom of wells, 50 μl HFRS antigen is added at a concentration four to eight times lower than its endpoint titer determined in previous tests (4 to 8 units). The antigen is incubated as mentioned above, the unabsorbed antigen is removed, and the wells are washed and dried. A 50 μl aliquot of test serum is added in dilutions 1 : 8 and higher. Controls are normal control serum without HFRS antibodies (negative) and specific positive reference-serum with known titer. After overnight incubation at 4° C (or for 2 hrs at 37° C), unadsorbed sera are removed and the plate washed and dried. To each well, 50 μl of the radioactive conjugate is added. The subsequent steps are analogous to these described for "double-sandwich" SPRIA.

The serum titer is expressed as the highest dilution that inhibits radioactivity bound by more than 50% as compared with two controls (Radioactivity of wells with normal antigen is considered 100% inhibition or blocking. Radioactivity of wells with normal serum plus standard antigen is considered as inhibition).

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4. Standard procedure of immune adherence hemagglutination test for antigen and antibody assays of Hantaviruses

When antigen sites on the biomembranes of a bacterial cell react with an antibody molecule, complement activation is initiated and the C3b component produced is bound with the polysaccharide of the membranes. C3b receptors of human erythrocytes may form polyvalent bonds with such C3b components, leading to the binding of erythrocytes on the bacterial cell surface (immune adherence (IA)).
In cases of viral antigen, the C3b component, leading to the agglutination of human erythrocytes through the bridging of C3b-covered virions (immune adherence hemagglutination (IAHA)). Thus, the principle of IAHA is common with the complement-fixation (CF) test. After the IAHA test was established in the poxvirus system (113) and employed with hepatitis B antigens (114), with modification, as the stabilization of hemagglutination pattern by dithiothretol, it became widely used for the titration of both viral antigens (115−117) and antibodies (118−121) with high sensitivity. Critical studies of antibody avidities in IAHA through the course of infection were carried out with rotavirus and togavirus systems (122), and the mechanism of IAHA reaction was elucidated as described above.

IAHA was employed for the comparative serology of Hantaan and related viruses by using the infected Vero-E6 cells (with or without heat inactivation at 56°C for 30 min.) as the antigen, and differentiation of serotypes among various Hantavirus strains became possible (123, 124).

A. Materials

The following materials are required to conduct the IAHA test :

(1) IAHA buffer is prepared with :

a. 5×VBS	40.0 ml
b. dw	160.0 ml
c. 2% gelatin solution in dw	0.2 ml
d. BSA	200.0 mg

(2) 5×VBS (veronal-buffered saline) is composed of

a. NaCl	85.00 g
b. barbital	5.75 g
c. barbital sodium	3.75 g
d. $MgCl_2.6H_2O$	1.02 g
e. $CaCl_2.2H_2O$	0.22 g
f. NaN_3	2.00 g

to which distilled water (dw) is added to form 2,000 ml solution.

(3) Fresh guinea pig serum (GPS), which may be stored at -80° C.

(4) Dithiothreitol solution (DTT), 3 mg/ml, which must be prepared fresh for each test (obtainable as IAHA Eizai Kit, Eizai Corp., Tokyo, Japan).

(5) Human type 0 erythrocyte (RBC) suspension, 0.4% in IAHA buffer.

a. Blood is collected from several candidate donors in volumes of 5 ml and the IAHA sensitivity is tested by box titration. The blood sample giving the highest titer is selected and stored at 4° C by mixing 5 ml blood with 20 ml Alsever's solution. RBC may be stored at 4° C for approximately 1 month. For the titration of animal sera human RBC of any type may be used.

b. RBC stock is diluted nearly 30 fold with IAHA buffer. RBC concentration in adjusted to 0.4%, measuring by the cyanomethemoglobin method (122).

(6) For antigen preparation, HFRS virus infected Vero E6 cell monolayers are harvested 14 days post infection (in the case of the SR 11 strain, any incubation time giving satisfactory antigen yield may be adopted) by being scraped with a spatula and suspended in 5 ml VBS for each bottle of infected cells. Cell suspension is sonicated for 60 sec, at 27 KHz, 100 W. Supernatant, after a centrifugation at 1,500 g for 10 min. is used as the antigen preparation for the IAHA and complement fixation (CF) tests. In the case of strains SR 11 and Hantaan virus 76-118, antigen titers against homologous antisera are approximately 1 : 16 (CF). Control antigen preparation made from uninfected cells should be provided.
(7) Reaction plates are U-bottomed, polystyrene microtiter plates with 96 wells.

B. Titration

For the box titration :
(1) The serum sample to be assayed is inactivated by heating at 56° C for 30 min. before the test.
(2) Serial two-fold dilutions of antigen and serum are made in volumes of 0.5 ml.
(3) 0.025 ml aliquots of antigen and serum dilutions are dropped and mixed in the well and kept at room temperature (25° C) for 60 min.
(4) 0.025 ml aliquot of 1 : 100 dilution of GPS in IAHA buffer is added and the reaction mixtures are further incubated at 37° C for 40 min.
(5) 0.025 ml of 3 mg/ml DTT solution and 0.050 ml of 0.4% RBC suspension are added, mixed, and reaction mixtures kept at room temperature (25 C) for 2 hrs or more.
(6) The hemagglutination pattern is read and recorded.

For antigen titration :
(1) A dilution of inactivated standard antiserum (SAS) is made in IAHA buffer containing approximately 10 IAHA antibody units.

(2) Serial two-fold dilutions of the antigen preparations on the plate are made in volumes of 0.025 ml.
(3) 0.025 ml aliquots of SAS dilution are prepared.
(4) 0.025 ml aliquots of antigen and serum dilutions are dropped and mixed in the well and kept at room temperature (25 C) for 60 min.
(5) 0.025 ml aliquot of 1 : 100 dilution of GPS in IAHA buffer is added and the reaction mixtures are further incubated at 37 C for 40 min.
(6) 0.02 ml of 3 mg/ml DTT solution and 0.050 ml of 0.4% RBC suspension are added, mixed, and reaction mixtures kept at room temperature (25° C) for 2 hrs or more.
(7) The hemagglutinatin pattern is read and recorded.

For antibody titration :
(1) A dilution of standard antigen preparation (SAG) is made in IAHA buffer containing 4 IAHA antigen units.
(2) Serum samples to be assayed are inactivated at 56° C for 30 min.
(3) Serial two-fold dilutions of the serum samples on the plate are made with diluters in volumes of 0.025 ml.
(4) 0.025 ml of SAG dilution are prepared.
(5) 0.025 ml aliquots of antigen and serum dilutions are dropped and mixed in the well and kept at room temperature (25° C) for 60 min.
(6) 0.025 ml aliquot of 1 : 100 dilution of GPS in IAHA buffer is added and the reaction mixtures are further incubated at 37° C for 40 min.
(7) 0.025 ml of 3 mg/ml DTT solution and 0.050 ml of 0.4% RBC suspension are added, mixed, and reaction mixtures kept at room temperature (25° C) for 2 hrs or more.
(8) The hemagglutination pattern is read and recorded. Control mixtures whether devoid of antigen or with standard negative serum should also be confirmed.

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5. Hemagglutination inhibition test in the diagnosis of HFRS

The difficulties encountered in propagating Hantaan virus in the laboratory are reflected in the varieties of serologic procedures that have been adopted for the diagnosis of HFRS. The immunofluorescent antibody (IFA) test became the standard serodiagnostic technique as an outgrowth of Lee and Lee's early work (1), in which they exploited the only available source of viral antigen, lung sections of infected animals. As Hantaan virus became better characterized and conditions for its propagation in cell culture and suckling mice (SM) described (106), these systems have been used to produce antigen for more easily standardized tests.

Hemagglutinating activity (HA) of Hantaan virus was first demonstrated in preparations of virions purified by gradient-density centrifugation (Schmaljohn C., Dalrymple J., personal communication). More practical and conventional HA antigens have been prepared from brains of infected suckling mice by sucrose-acetone extraction and from infected cell culture (125–127). An excellent correlation between antibodies detected by IFA and hemagglutination inhibition (HI) has been observed, and the tests offer equal sensitivity. HI antibody is long lived and remains elevated in patients 20 years after onset of HFRS, so that the technique is applicable for cross-sectional serosurveys. Rerults of HI determinations are far more specific than measurement of IF antibody. In areas where hantaviruses co-circulate, HI is recomended as the serologic test of first choice.

The practical disadvantages of the IFA test – the expense of a fluorescence microscope, the need for antispecies conjugates, and the subjectivity in interpreting reading – are obviated by the HI test.

A. Preparation of hemagglutinating antigen

Maintenance of procedures ensuring the safety of laboratory workers is paramount. Manipulations of infectious virus should be carried out in a safety cabinet. For optimal protection, the antigen should be inactivated before homogenization and extraction, when aerosols are most likely to be generated. Alternatively, the antigen can be inactivated after extraction, although the effects of β-propiolactone and ultraviolet irradiation on HA activity have not been evaluated.

Suckling mice (2 to 5 days old) are inoculated intracerebrally with 10^2 to 10^3 $SMLD_{50}$ of a Hantaan virus strain previously adapted to mice (106). The mice are harvested 10 to 14 days after inoculation or when 10% of the animals have died. Brain is aspirated from thawed, previously frozen animals and inactivated at this point by radiation from a ^{60}Cobalt source. The brain will have an infectious titer of approximately 10^9 $TCID_{50}/g$ and should be irradiated at 4°C with 10^6 rads ; if possible, the material is tested for the presence of viable virus before proceeding further.

The brain is homogenized and extracted as described by Clarke and Casals (128). The dried precipitate is reconstituted overnight in normal saline, sonicated in the cold at low intensity (300 w), and centrifuged (500 g for 10 min). The supernatant fluid is the antigen. A control antigen is prepared in the same fashion from brains of uninfected mice of the same age as the harvested infected mice.

An HA antigen can be prepared from infected Vero-E6 cell cultures. Five to seven days after inoculation, the supernatant fluid is discarded, and the cell monolayer is rinsed and refed with a serum-free medium containing 0.4% bovine serum albumin (BSA). The fluid phase is harvested 8 to 10 days later and clarified by low-speed centrifugation. Viral proteins are precipitated by adding polyethylene glycol (MW 6,000) and NaCl (final concentrations 8% and 0.5 M, respectively). The medium is stirred for several hours or overnight in the cold and centrifuged (10,000 g) for 30 min. The pellet is

resuspended in borate buffered saline, pH 9.0, so that the final volume of antigen is one hundredth the starting volume of cell culture fluid. The HA titer of a Prospect Hill virus antigen prepared in this fashion is 1 : 256.

The titer of the cell culture derived antigen can be increased further by extracting it with acetone and ether and sonicating the extracted antigen (127), alternatively a non-infectious antigen, can be prepared from cell culture fluid by tween-ether extraction (126).

B. Hemagglutination test

A standard microtiter procedure for arbovirus HA determination is used (129). HA antigens prepared from all four hantaviruses (Hantaan, Seoul, Prospect Hill, and Puumala viruses) hemagglutinate gander (male goose) erythrocytes optimally between pH 5.8 & 6.0. Hemagglutination occurs at either room temperature (20 C) or 37° C. The antigen is titrated before each HI test to determine the optimum pH for the test and the dilution of antigen that will yield 4 to 8 HA units. Like many arbovirus HA antigens, the HA titer of the antigen immediately after thawing may be low, but increases after standing at 20° C for several hours.

C. Hemagglutination inhibition test

Sera are extracted twice with acetone and absorbed with packed goose erythrocytes. A standard microtiter procedure is used, in which two-fold serum dilutions are tested beginning at 1 : 10. Serum and 4 to 8 units of antigen are incubated for 2 hr at room temperature or overnight at 4° C. Gander erythrocytes are added, and hemagglutination patterns may be read after 30 min.

D. Sensitivity

The sensitivity of the HI test has been evaluated by comparing HI and IFA antibody titers in sera from epidemic hemorrhagic fever and nephropathia epidemica patient. IFA and HI antibody titers were significantly correlated ;

the HI test was ≧97% sensitive (125, 126).

HI antibody has been detected in sera from laboratory rats, *Apodemus agrarius* and *Suncus murinus* (125), and the procedure should have widespread applicability in screening other animals for evidence of Hantaan virus-related infections.

E. Specificity

No hemagglutination inhibition was observed in NIAID, polyvalent arbovirus grouping fluids tested against a Hantaan virus antigen, confirming experience with other serologic procedures which failed to show an antigenic relationship of Hantaan virus with other Bunyaviridae.

Cross-HI tests with antigens prepared from Hantaan-related viruses have shown that heterologous reactions occur among Hantaan, Seoul, Puumala and Prospect Hill viruses but that heterologous reactions are sufficienthy distinct i.e. ≧4 fold difference, that infections from Puumala, Hantaan and Seoul viruses can be distinguished (126, 127).

In laboratories lacking cell culture capabilities of the containment facilities requrired to work with infectious virus, HI may be a satisfactory alternative to the neutralisation test as a means of distinguishing among hantavirus infections.

F. Antibody response

In serial blood samples from the few patients who have been examined, HI antibody was present in all acute sera tested. HI antibody appears to be long lived - the geometric mean titer of 13 patients bled 10 to 20 years after recovery from HFRS was 1 : 146. However, the possibility that antibody was boosted by re-exposure cannot be excluded (128).

The HI test is a sensitive and specific procedure for the diagnosis of HFRS. It is simple, familiar to most virologists, inexpensive to perform, and applicable for both the diagnosis of human infection and for animal serosurveys. The test is the most convenient means of identifying the specific cause of a hantaviral infection.

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6. Plaque assay and plaque-reduction neutralization tests

Hantaan virus, strain 76-118, has been shown to produce plaques on cell culture monolayers following the adaptation of this virus to replication in cell culture (130). In fact, the reference virus available from the American Type Culture Collection (ATCC VR 938) was plaque purified by the repeated selection of virus producing large plaques prior to preparation of the seed virus preparation (48). More recent data suggest that most, if not all, isolates of Hantaan-like viruses produce plaques once they have been adapted to replication in cell culture. The following procedures have been most successful in the plaque assay of these agents.

The prototype strain of Hantaan virus was originally adapted to replicate in the A549 cell line (ATCC CCL 185). Although Hantaan virus has been shown to produce plaques on these A549 cells, as well as LLC-MK2 cells (ATCC CCL 7). The cell of choice for the plaque assay of Hantaan and related viruses is the Vero-E6 cell line. These cells represent a clone (C1008) of the parent Vero-E6 cells, selected by Price (131) in 1979, and are available as ATCC CRL 1586, from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA (These cells are described in detail on pages 145 and 146 of the ATCC catalog). These cells have the ability of being maintained as a confluent monolayer for long periods without significant deterioration of the cell sheet. The selection of this cell line appears to be important for the plaque assay of Hantaan virus, since plaques have not been demonstrated when using the parent Vero cell line. It should be emphasized

that cell culture-adapted Hantaan and related viruses will replicate in a variety of cell lines that have not been shown to be good substrates for the plaque assay of these viruses.

Cells are propagated to a confluent monolayer in either T-25 sealed flasks or 12-well plates, which must be incubated in an atmosphere containing 5% CO_2. Cells are propagated in Eagle's Minimum Essential Medium (EMEM) containing nonessential amino acids and supplemented with glutamine, 10% heated fetal bovine werum, and antibiotics (growth medium). A T-25 flask inoculated with 5 ml or a single well of a 12-well plate inoculated with 2 ml containing 2 to 3×5^{10} cells/ml should form a confluent monolayer within 3 to 5 days incubation at 37°C. Although not precisely defined by detailed experimentation, it appears that better plaques are obtained on older cells that are totally confluent as opposed to younger, subconfluent monolayers.

Virus suspensions to be assayed are diluted in growth medium and are added to decanted cell monolayers (0.1 or 0.2 ml) and allowed to adsorb for 60 to 90 min. at 37° C in a humid, 5% CO^2 atmosphere. An agarose overlay is added following virus adsorption and allowed to harden at room etmperature before cultures are returned to the incubator. The composition of the agarose overlay is listed in Table 10.

The incubation time required for mixiumum plaque development is variable, depending on the virus isolate and passage history in Vero-E6 cells. Early experience suggested that 10 to 14 days were required for Hantaan-virus plaques to develop, but this virus now produces good plaques in 6 to 8 days. Certain Hantaan-virus strains produce plaques more rapidly at slightly elevated temperatures (38° C to 38.5° C) ; however, other viruses such as Puumala virus (Nephropathia epidemica) still require 12 to 16 days incubation at 37° C, with higher temperatures actually decreasing plaque clarity. Care should be exercised in experimenting with elevated temperatures because the Vero-E6 cells do not survive well

above 39° C.

Staining of the monolayer is accomplished by the addition of a second agar overlay containing stain. The composition of this second overlay is essentially the same as that described in Table 10, except that the fetal bovine serum can be reduced and a commercial neutral red solution* added to a concentration of 5% (16.7 mg/10 ml final concentration).

Table 10. Composition of agarose overlay for Hantaan virus plaque assay.

Ingredient	Amount/100 ml
Agarose[1]	0.06 gm
Double distilled water[2]	34.0 ml
(Autoclave to sterilize and facilitate solution, cool to 42 C and add remaining ingredients prewarmed to 42 C)	
Fetal bovine serum[3]	10.0 ml
L-glutamine (200 mM)[4]	4.0 ml
2 X concentrated MEM with Eagle's salts[5]	50.0 ml
100 X nonessential amino acid mixture[6]	1.0 ml
Antibiotics[7]	

1. Sea Kem agarose (ME), Marine Colloids Division, FMC Corporation, P. O. BOX 308, Rockland, Maine 04841, USA.
2. Purified water suitable for use in cell culture.
3. Fetal bovine serum previously heated at 56 C for 30 min.
4. L-glutamine (200 mM = 100 X), #320-5030, Gibco, 3175 Stanley Road, Grand Island, New York 14072, USA.
5. MEM-Eagle (2 X), #12-668A, M.A. Bioproducts, Building 100, Biggs Ford Road, Walkersville, Maryland 21793, USA.
6. MEM nonessential amino acids (100 X), #320-1140, Gibco, 3175 Stanley Road, Grand Island, New York 14072, USA.
7. Antibioties used for cell culture, generally penicillin and streptomycin at 100 units/ml and 100 μg/ml final concentration.

*#630-5330 Neutral red solution, 333.0 mg/l neutral red (Sodium salt), Gibco, 3175 Stanley Road, Grand Island, New York 14072, USA.

Following the addition of the staining overlay, the cells are returned to the incubator, and plaques can frequently be counted the following day. Additional incubation is often required to improve plaque clarity with less well-adapted

viruses.

Plaque-reduction neutralization tests are routinely employed to examine suspect sera for neutralizing antibody and to confirm a diagnosis of HFRS. This test is performed by mixing equal volumes of serum dilutions with a virus suspension previously titrated by plaque assay. The virus dilution is adjusted such that the resultant 1 : 2 dilution obtaine by mixing equal volumes should yield approximately 100 plaques. in the absence of any neutralization. Sera are generally heated at 56° C for 30 min. prior to dilution in growth medium and the virus-serum dilution mixtures are incubated at 37° C for 1 hr prior to performing the plaque assay. Incubations of 12 to 18 hrs at 4° C have not significantly increased the sensitivity of this test. The neutralization endpoint titer of each serum tested is recorded as the highest dilution of serum resulting in a reduction in the number of plaques by 80% or greater. In practice, sera are frequently screened at large dilution intervals (ten-fold) and the test repeated over more narrow dilution intervals (two-fold) to more precisely define the endpoint.

The plaque-reduction neutralization test is the most specific serologic procedure examined at Ft. Detrick. It is most useful in differentiating virus strains that are closely related to Hantaan virus. The plaque reduction neutralization comparison of eight HFRS agents using single-injection, laboratory rat immune sera is shown in Table 11. These data are shown as an example of the specificity of the test in distinguishing different isolates.

Many investigators have reported difficulties with the plaque assay and plaque-reduction neutralization testing of these agents. The authors' experience would suggest that the procedures described above work well for Hantaan (76-118) and that all of the agents listed in Table 10 will plaque readily with only slight modifications, such as the increased incubation time required with Puumala virus. It should be emphasized that all agents described required adaptation to replication in cell culture before plaques could be observed in

this assay.

Table 11. Plaque reduction neutralization of Hantaviruses.

Serum pools	Hantaan virus 76-118	Hantaan virus Lee	Seoul virus	Sapporo rat	Tchoupitoulas	Girard Point	Prospect Hill	Puumala
Hantaan (76-118)	4,000	4,000	–	40	160	80	–	–
Lee (human)	4.000	2,000	20	200	160	160	–	–
Seoul virus	2,000	80	4,000	8,000	16,000	16,000	160	–
Sapporo rat	2,000	20	2,000	2,000	2,000	2,000	200	–
Tchoupitoulas	2,000	200	4,000	8,000	32,000	8,000	200	–
Girard Point	200	80	2,000	8,000	8,000	8,000	200	–
Prospect Hill	–	–	–	20	–	20	4,000	–
Puumala (NE)	–	–	–	–	–	–	–	1,280

Titers expressed as reciprocal of the highest dilution of antisera with greater than 80% reduction of approximately 100 plaques.
– =Titer less than 20.

Chapter IX

Laboratory Safety

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Laboratory Safety

Hantaan virus aerosols are highly infectious for humans. This review of laboratory safety deals specifically with that risk.

Clinical specimens from patients with HFRS pose a limited biohazard. Only in a few instances has virus been isolated from blood or secretions, and only then from patients sampled early in the course of illness prior to appearance of high-titered antibodies. Most patients are antibody positive upon hospital admission.

Tissues and excreta from wild rodents, in contrast, may contain Hantaan-related viruses, sometimes in significant quantities. It is imperative that such materials are manipulated in a hood designed to capture aerosols produced by procedures such as grinding or blending.

1. Virus inactivation

In the liquid state, Hantaan virions are readily inactivated by any of the following methods :

(1) chloroform
(2) ether-alcohol
(3) β-propiolactone
(4) heat 60° C, 30 min
(5) acid, below pH 5.0
(6) phenol
(7) sodium hypochloride
(8) ^{60}cobalt irradiation

Virus infected cells placed on glass slides to prepare antigens for immunofluorescent antibody tests is apparently inactivated by immersion in pure acetone for 10 min.

2. Aerosol hazards

A. Infected animals

Certain rodents (*Apodemus, Rattus, Clethrionomys, Microtus*)

can be infected *chronically* with Hantaan or related viruses. Virus may be shed for weeks or months in urine, saliva, and feces. Both intracage and intercage virus transmission has been documented, and many laboratory personnel have been infected while working with rodents known, or more often not known, to be infected.

B. Infected cells

Strains of Hantaan-related viruses adapted to replicate well in cell cultures pose a series of aerosol hazards because virus concentrations achieved far exceed those encountered in nature. These concentrations are frequently increased as much as 100 times in the course of morphologic or biochemical investigations. For this reason, it is recommended that all work that might generate aerosols be done in a Class Ⅱ laminar flow biosafety hood, or its equivalent.

3. Recommended facilities and procedures

Facilities and procedures recommended by WHO for Biohazard level 3 containment should be used when working with Hantaan-related viruses. In addition, the following special precautions should be taken :

(1) Integrity of contrifuge *rotors* must be maintained at all times. Broken tubes can produce tremendous aerosols. Open rotors should only be used in a hood.

(2) Integrity of laminar flow hoods also must be maintained. Micro centrifuges, blenders, etc., may disrupt the air curtain depending on location in the hood. Test new instrument in the hood before using with virus.

(3) Personnel working in rooms with infected animals *must* have complete protection from aerosols. Animals may be kept in filtered cage racks or cages and high-efficiency respirators should be worn at all times. Animal bedding should be autoclaved in the cages if possible.

Chapter X

Infection and Control of Hantaan and Related Virus in Laboratory Rodent Colonies

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Infection and Control of Hantaan and Related Virus in Laboratory Rodent Colonies

An outbreak of a febrile disease with renal involvement in Japan was made public in November 1978. Only those who had close contact with laboratory rats were affected. The disease resembled KHF found in Korea or epidemic hemorrhagic fever that was prevalent in the Osa, area from 1960 to 1970. The disease was finally diagnosed serologically by Lee (24) as Hemorrhagic fever with renal syndrome (known as Hantaan virus) by the indirect immuno fluorescent antibody (IFA) technique. A retrospective survey traced the occurrence of laboratory type Hantaan-like virus back to 1970. From 1970 until the end of March 1986, 126 cases of Seoul virus infections were documented in universities and institutes. One fatal case occurred in a medical college in 1981 (25, 73). Laboratory acquired infections were also repo ted in Korea, China, and Europe (26-28, 85-88).

Serologic testing of laboratory rats received from animal facilities of universities and institutes in Japan revealed that in 1979, 89.5% of the rat sera were IFA positive. In 1982, 50% of rat sera tested were IFA positive and infection of laboratory rats is declining. The rat of the facility where the fatality occurred demonstrated an exceptionally high IFA titer. The laboratory rats of Japan were contaminated by some unknown means, supposedly by accidental contact with contaminated wild rodents in a breeding farm or in a laboratory.

Control of laboratory animal infection by Hantaan related viruses is only possible by eradication of all infected animals

from the laboratory animal facility and introduction of uninfected, clean laboratory animals. Consequently, continuous monitoring of laboratory animals by IFA for Hantaan-related viruses is required both in animal facilities and laboratory animal breeding farms.

1. Transmission in laboratory rodent colonies

Transmission of Hantaan related viruses from infected rats to humans in the laboratory setting is thought to be via aerosol. Infectious virus has been found in urine, feces, and saliva of infected rats. Localization of antigen has been demonstrated in most organs, including lungs, pancreas, parotids, intestines, and kidneys. Infected rats shed infectious virus for extended periods. Persistence of infectious virus in animal bedding has not been established, but should be considered a potential hazard pending additional studies. Surgery on infected rats could be extremely hazardous due to inhalation of aerosols or accidental wounds incurred during manipulation of the animals.

Hantaan or related viruses also can be transmitted from one rat to another by transplantation of tissue fragments from a rat tumor obtained from infected donor rats. Another source of infection includes laboratory animals other than rats, such as mice, Mongolian gerbils, and hamsters, which were proven (73) by serologic testing to be potential sources of laboratory infection of Hantaan or related viruses.

2. Diagnosis

Hantaan or related virus infection is diagnosed by demonstration of specific anti-Hantaan virus antibody in infected hosts. Large-scale screening is best conducted using the immunofluorescent antibody (IFA) technique. Selection of a suitable virus strain is essential and should include a laboratory rat virus isolated from the area whenever possible. Antigen preparation should be carefully controlled to obtain

reproducible results. Characteristic Hantaan or related viral antigen can be detected in organs or tissues by immunofluorescence.

Isolation of etiologic virus from infected laboratory rats can be achieved by inoculation of tissue fragments (lungs, tumor tissue) obtained from infected animals with high titered anti-Hantaan antibody on Vero-E6 or A549 (human lung cancer cells derived) cell monolayers. Subsequent rodent-to-rodent and cell-to-cell passage is also possible.

3. Prevention and control of laboratory transmission

No vaccine is available for Hantaan virus yet, although studies are in progress. Nevertheless, nine steps can be taken to prevent and control the spread of Hantaan or related virus infections in a laboratory setting :

(1) Hantaan virus-free colonies are available only when the animals are repeatedly tested by IFAT and breeding started from virus-free parents.

(2) Hysterectomy derivation to obtain Hantaan virus-free colonies may have merit, though well-controlled studies to evaluate its efficacy have not been conducted.

(3) Breeders and suppliers should monitor laboratory animals for infection using IFA technique against Hantaan virus before shipment.

(4) Laboratory animal facilities should make certain that facilities where the animals are bred or maintained have well-controlled, sanitary conditions and periodically monitor for Hantaan or related viruses.
Arriving animals should be quarantined for a limited period and tested by IFAT against Hantaan virus.

(5) To protect against inhalation of contaminated aerosol originated from bedding or excreta of the animals, protective caps, masks, gloves, gowns, and boots should be worn in the animal area. Washing equipment and laboratory workers with chemical disinfectants is

effective. Clothing is best sterilized by autoclaving or ultraviolet light.

(6) To prevent accidental contamination of laboratory animals, housing of the appropriate number of laboratory animals in a room is essential. An overcrowded animal room often promotes Hantaan virus infection of humans from contaminated laboratory animals.

(7) Constant cleaning and sterilization both in and outside the facility are particularly necessary. Elimination of wild rodents and a good pesticide management program are also required. Other essential points to be monitored are heating and air conditioning units and the airflow of the laboratory animal facility.

(8) Exhaustion provides a predisposing physical condition for Hantaan virus infection. Maintaining good health of animal care and experimental staff is an important means to avoid infection.

(9) If a laboratory worker or animal demonstrates a high IF antibody against Hantaan virus, all animals in the facility may be highly contaminated. In such a case, the rooms can be best sterilized by eliminating all the animals and the rooms completely disinfected.

Chapter XI

Standard Reference Reagents

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Standard Reference Reagents

Worldwide reference reagents, including monoclonal antibodies, and standards for the Hantaan-related viruses are not yet available. Nevertheless, procedures may be outlined that will allow each laboratory working with this group of viruses to make standards reagents using either the prototype Hantaan 76-118 or another, well-characterized strain. These standard reagents may then be compared to products currently undergoing testing as potential reference reagents.

1. Standard reagents

Two types of standard reagents are presently produced by several laboratories. The first and simplest is polyclonal mouse ascitic fluid, and the second is monoclonal mouse ascitic fluid.

A. Polyclonal mouse ascitic fluid

Animal work with Hantaan or related viruses must be carried out in a high-containment laboratory or in a well-contained laboratory (level 3) by immune personnel since human infection from laboratory animals is a well-recognized risk. If such conditions are not available, then inactivated antigen may be obtained from another laboratory and used under less stringent circumstances.

Antigen is prepared by inoculating Hantaan virus (e.g., strain 76-118) into suckling mice intracerebrally and harvesting the brains after 10 to 12 days. The material is homogenized and stored either without dilution until needed or a 10% suspension made in phosphate-buffered saline for inoculation into 5-to 6-week old mice.

If adequate containment facilities are available, the suspension material is mixed with Freund's adjuvant (equal quantities) and 0.2 ml injected intraperitoneally (IP). This is followed by two more inoculations without adjuvant every 7 days. A fourth dose is given 3 days later, and sarcoma cells may be injected after another 3 days. Ten days after the sarcoma cells are given, ascites may be harvested daily for 4 to 7 days.

If adequate containment facilities are not available, the brain suspension may be inactivated by 0.1% formalin for 3 to 4 days at 4° C, by gamma irradiation (2.4 to 2.8 million rads in a cobalt-60 cell), or by BPL. This material may then be inoculated into the mouse peritoneum as described above.

Ascitic fluid is held for 1 to 3 hr at room temperature, and the clots are broken up with a pipette.

The suspension is clarified by spinning at 15,000 g for about 1 hr. The clarified ascitic fluid may then be stored at 4° C until further clarification by treatment with 2 M glacial acetic acid, which eliminates the problem of further clot formation. The material is further clarified by centrifugation as above and stored in aliquots at -20° C. Alternatively, the ascitic fluid may be lyophilized for long-term storage.

B. Monoclonal antibody

Preparation of monoclonal antibodies is a time-consuming procedure, and it may not be necessary unless antibodies with specified characteristics or those describing new virus strains are desired. A limited supply of antibodies or hybridoma cells is available from the Special Pathogens Branch, Division of Viral Diseases, Center for Disease Control, Atlanta, Georgia, or the Virology Division, USAMRIID, Ft. Detrick, Frederick, Maryland, USA.

C. Antigen preparation for monoclonal antibodies

Antigen is prepared in Balb/c mouse brain as described above. The material is made into a 20% (by volume)

suspension in PBS and emulsified with and 18-gauge needle. The antigen is inactivated using one of the three methods described. Safety testing is done by inoculating serial dilutions of brain suspension into Vero-E6 cells in roller tubes, incubating 10 days, and assaying for intracellular antigen by immunofluorescence. Balb/c mice are inoculated ip initially with 0.5 ml of a 1 : 1 suspension of inactivated brain material and adjuvant. Subsequent injections of 0.1 to 0.2 ml brain suspension (without adjuvant) are given ip, subcutaneously, or intravenously (IV) every week over the course of 3 weeks to 5 months, depending on the antibody response. An IFA titer of 1 : 256 or greater is considered a good response. A final IV injection of antigen is given before the spleen is harvested 5 days later. The production of hybridomas is accomplished with standard published methods.

D. Ascitic fluid monoclonal antibody production

Since hybridoma cells are available, some laboratories may want to produce their own ascitic fluids. Adult Balb/c female mice are inoculated ip with 0.5 ml pristane and left for 3 weeks. Approximately 5×10^5 hybridoma cells are inoculated ip, and ascitic fluid is collected beginning on the 8th to 10th days after injection of the cells. Ascitic fluid may be clarified, as described above, and stored frozen or lyophilized.

E. Standard antigen reagents

There are presently three methods of producing antigen for use in serologic testing : tissue culture, mouse brain, or *Apodemus* or *Clethrionomys* lung. For most serologic tests, the use of tissue culture has or is replacing the other methods. Production of working reagents is amply described in this manual. In general, any laboratory wanting to produce its own working reagents would be wise to request one of the prototype strains with relevant data from a WHO collaborating center and make a pool of material that can be used for comparison with other viral strains with which they

wish to work.

All of the working reagents produced should be standardized and titrated with one or more of the tests described in this manual, depending on their intended use. Although no international reference reagents are presently available, comparison reagents are available from several of the WHO collaborating centers, and these may be obtained for comparison with locally produced, working or standard reagents by any of the tests described in this manual. Such comparative tests should be performed simultaneously under identical laboratory conditions in order to be reliable. Monoclonal antibodies should be characterized as to their monoclonality, IgG subclass, and viral protein specificity before they are widely used and distributed.

2. Shipping

A. Air freight

Biologic materials must be packaged according to the guidelines established by the International Air Transport Association regulations. This generally requires that materials be placed in watertight primary and secondary containers, which are then placed in an insulated box containing dry ice.

The transit time is the most important factor determining the quantity of dry ice to be used in a frozen shipment. It is, therefore, critical to know the air schedule prior to packaging the material to be shipped. It is also important to schedule the material with the airline just as one would schedule a passenger. This will help ensure timely arrival of the material with minimal loss. Shippers should adhere to the following procedures :

(1) Prepare a shipping/packing list in duplicate.
(2) Place one copy in the package, retain one in the shipping laboratory.
(3) Send a cable to the addressee giving the airline, flight number, the estimated day and time of arrival, and the

airway bill number.

B. Air mail

Only nonperishable material (e.g., serum, lyophilized material) should be sent air mail, and the following procedures should be followed :

(1) Package must conform to the size and weight regulations of the Postal Service.
(2) Contact the Postal Service to ascertain rules and regulations of country to which material is being shipped.

References

1. Lee HW, Lee PW : Korean hemorrhagic fever. I. Demonstration of causative antigen and antibodies. Kor J Intern Med 19 : 371 383, 1976
2. Lee HW, Lee PW : Korean hemorrhagic fever. II. Isolation of etiologic agent. J Kor Soc Virol 7 : 1 9, 1977
3. Lee HW, French GR, Lee PW, Baek LJ, Tsuchiya K, Foulke RS : Observations on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever. Am J Trop Med Hyg 30 : 477 482, 1981
4. Lee HW, Lee PW, Johnson KM : Isolation of the etiologic agent of Korean hemorrhagic fever. J Infect Dis 137 : 298-308, 1978
5. Lee PW, Gajdusek DC, Gibbs CJ, Xu ZY : Aetiological relation between Korean haemorrhagic fever and epidemic haemorrhagic fever with renal syndrome in the People's Republic of China. Lancet 1 : 819 820, 1980
6. Lee PW, Gibbs CJ, Gajdusek DC, Hsiang CM, Hsiung GD : Identification of epidemic haemorrhagic fever with renal syndrome in China with Korean haemorrhagic fever. Lancet 1 : 1025 1026, 1980
7. Cohen MS, Casals J, Hsiung GD, Kwei HE, Chin CC, Ge HC, Hsiang CM, Lee PW, Gibbs CJ, Gajdusek DC : Epidemic hemorrhagic fever in Hubei province, the People's Republic of China : a clinical and serological study. Yale J Biol Med 54 : 41 55, 1981
8. Lee PW, Svedmyr A, Gajdusek DC, Gibbs CJ, Nystrom K : Antigenic difference between European and East Asian viruses causing haemorrhagic fever with renal syndrome. Lancet 2 : 256 257, 1981
9. Svedmyr A, Lee PW, Gajdusek DC, Gibbs CJ, Nystrom K : Antigenic differentiation of the viruses causing Korean haemorrhagic fever and epidemic (endemic) nephropathy of Scandinavia. Lancet 2 : 315-316, 1980

10. Lee PW, Goldgaber D, Gibbs CJ, Gajdusek DC, Yanagihara R, Svedmyr A, Hlaca D, Vesenjak-Hirjan J, Gligic A : Other serotypes of haemorrhagic fever with renal syndrome viruses in Europe. Lancet 2 : 1405-1406, 1982
11. Brummer-Korvenkontio M, Vaheri A, von Bonsdorff CH, Vuorimies J, Manni T, Penttinen K, Oker-Blom N, Laehdevirta J : Nephropathia epidemica : detection of antigen in bank voles and serologic diagnosis of human infection. J Infect Dis 141 : 131-134, 1980
12. Brummer-Korvenkontio M, Henttonen, H, Vaheri A : Hemorrhagic fever with renal syndrome in Finland : ecology and virology of nephropathia epidemica. Scand J Infect Dis Suppl 36 : 88-91, 1982
13. Yanagihara R, Svedmyr A, Amyx HL, Lee PW, Goldgaber D, Gajdusek DC, Gibbs CJ, Nystrom, K : Isolation and propagation of nephropathia epidemica virus in bank voles. Scand J Infect Dis 16 : 225-228, 1984
14. Lee HW, Bark DH, Baek LJ, Choi KS, Whang YN, Woo MS : Korean hemorrhagic fever in urban areas of Seoul. J Kor Soc Virol 10 : 1-6, 1980
15. Lee HW, Baek LJ, Johnson KM : Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J Infect Dis 146 : 638-644, 1982
16. Tamura M : Occurrence of epidemic hemorrhagic fever in Osaka City : first cases found in Japan and characteristic feature of marked proteinuria. Biken J 7 : 79-94, 1964
17. Sugiyama K, Morita C, Matsuura Y, Shiga S, Komatsu Y, Morikawa S, Kitamura T : Isolation of a virus related to hemorrhagic fever with renal syndrome from urban rats in a nonendemic area. J Infect Dis 149 : 473, 1984
18. Song G, Hang CS, Qui XZ, Ni DS, Liao HX, Gao GZ, Du YL, Xu JK, Wu YS, Zhao JN, Kong BX, Wans ZS, Shang ZQ, Shen HK, Zhou N : Etiologic studies of epidemic hemorrhagic fever (hemorrhagic fever with renal syndrome). J Infect Dis 147 : 654-659, 1983
19. LeDuc JW, Smith GA, Johnson KM : Hantaan-like viruses from domestic rats captured in the United States. Am J

Trop Med Hyg 33 : 992-998, 1984
20. Tsai TF, Bauer SP, Sasso DR, Whitfield SG, McCormick JB, Caraway TC, McFarland L, Bradford H, Kurata T : Serological and virological evidence of a Hantaan virus-related enzootic in the United States. J Infect Dis 152 : 126-136, 1985
21. Childs JE, Korch GW, Glass GE, LeDuc JW, Shah KV : Epizootiology of Hantavirus infections in Baltimore : isolation of a virus from Norway rats, and characteristics of infected rat populations. Am J Epid 126 : 55-68, 1987
22. LeDuc JW, Smith GA, Pinheiro FP, Vasconcelos PFC, Rosa EST, Maiztegui JI : Isolation of a Hantaan-related virus from Brazilian rats and serological evidence of its widespread distribution in South America. Am J Trop Med Hyg 34 : 810-815, 1985
23. Yanagihara R, Chin CT, Weiss MB, Gajdusek DC, Diwan AR, Poland JB, Kleeman KT, Wilfert CM, Meiklejohn G, Glezen WP : Serological evidence of Hantaan virus infection in the United States. Am J Trop Med Hyg 34 : 396 399, 1985
24. Umenai T, Lee HW, Lee PW, Saito T, Toyoda T, Hongo M, Hoshinaga K, Nobunaga T, Horiuchi T, Ishida N : Korean haemorrhagic fever in an animal laboratory. Lancet 1 : 1314-1316, 1979
25. Kawamata J, Yamanouchi T, Dohmae K, Takahashi M, Yamanishi K, Miyamoto H, Lee HW : Recent status and control of laboratory type infection of hemorrhagic fever with renal syndrome (HFRS) in Japan. In : Proceedings of the 8th ICLAS/CALAS Symposium, Vancouver, New York : Gustav Fischer Verlag, pp. 267-272, 1983
26. Desmyter J, LeDuc JW, Johnson KM, Brasseur F, Deckers C, van Ypersele de Strihou C : Laboratory rat associated outbreak of haemorrhagic fever with renal syndrome due to Hantaan-like virus in Belgium. Lancet 2 : 1445 1448, 1983
27. Lee HW, Johnson KM : Laboratory-acquired infections with

Hantaan virus, the etiologic agent of Korean hemorrhagic fever. J Infect Dis 146 : 645-651, 1982
28. Lloyd G, Bowen ETW, Jones N, Pendry A : HFRS outbreak associated with laboratory rats in UK. Lancet 1 : 1175-1176, 1984
29. Laehdevirta J : Nephropathia epidemica in Finland. A clinical, histological and epidemiological study. Ann Clin Res 3 Suppl 8 : 1-154, 1971
30. Lee PW, Gibbs CJ, Gajdusek DC, Svedmyr A : Antibody to Korean haemorrhagic fever virus in man in parts of the world where haemorrhagic fever with renal syndrome is not known. Lancet 2 : 256, 1980
31. Tkachenko EA, Dzugurova TK, Leshchinskaya EV, Zagidulin IM, Ustijugova IM, Gasanova TA, Rezapkin GV, Miasnikov JA : Serological diagnosis of haemorrhagic fever with renal syndrome in European regions of U.S.S.R. Lancet 2 : 1407, 1982
32. Lee PW, Amyx HL, Yanagihara R, Gajdusek DC, Goldgaber D, Gibbs CJ : Partial characterization of Prospect Hill virus isolated from meadow voles in the United States. J Infect Dis 152 : 826-829, 1985
33. Yanagihara R, Gajdusek DC, Gibbs CJ, Traub R : Prospect Hill virus : serological evidence for infection in mammalogists. N Engl J Med 310 : 1325-1326, 1984
34. Yanagihara R, Gajdusek DC : Hemorrhagic fever with renal syndrome : a historical perspective and review of recent advances. In : Gear, J.H.S., ed. CRC Handbook of Viral and Rickettsial Hemorrhagic Fevers. CRC Press Inc Baca Raton Gla pp 155-181, 1988
35. Gajdusek DC : Muroid virus nephropathies and muroid viruses of the Hantaan virus group. Scand J Infect Dis Suppl 36 : 96-108, 1982
36. Smorodintsev AA, Chumakov VG, Churilov AV : Haemorrhagic nephrosonephritis. New York : Pergamon Press pp 124, 1959
37. Kasahara S, Kitano M, Kikuchi H, Sakuyama M, Kanazawa K, Nezu N, Yoshimura M, Kudo T : Ryukosei shukketsu

netsu no byogentai no kettei (Studies on the pathogen of epidemic hemorrhatic fever). Nihon Byori Gakkai Zasshi (J Jap Patho) 34 : 3-5, 1944
38. Kitano M : Ryukosei shukketsunetsu ni kan-suru kenkyuu (Studies on epidemic hemorrhagic fever). Manshu Igaku Zasshi (Manchoukuo Med J) 40 : 191-209, 1944
39. Smorodintsev AA, Al'tshuler IS, Dunaevskii ML, Kiselev MV, Churilov AV, Darshkevich V : (Clinical symptomatology of experimental hemorrhagic nephroso-nephritis). Chapter 6 in : Smorodintsev, AA, ed.. Etiology and clinical aspects of hemorrhagic nephrosonephritis. Medgiz Moscow pp 38-46, 1944
40. Smadel JE : Epidemic hemorrhagic fever. Am J Publ Hlth 43 : 1327-1330, 1953
41. Sheedy JA, Froeb HF, Batson HA, Congley CG, Murphy JP, Hunter RB, Gugell DW, Giles RB, Bershadski SC, Vester JW, Yoe RH : The clinical course of epidemic hemorrhagic fever. Am J Med 16 : 619-628, 1954
42. United States Armed Forces, Department of the Army. Epidemic haemorrhagic fever. Technical Bulletin TB MED 240 U.S. Government Printing Office Washington DC pp 23, 1962
43. Gajdusek DC : Virus hemorrhagic fevers. Special reference to hemorrhagic fever with renal syndrome (epidemic hemorrhagic fever). J Pediatr 60 : 841-857, 1962
44. Chiu FS, Hou TC, Huang PS, Liu WC, Sung HY, Li Y : Investigations into epidemic hemorrhagic fever in Inner Mongolia. A clinical study of 104 cases. Chung Hua Nei Ko Tsa Chih (Chin J Intern Med) 5 : 349-361, 1957
45. Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, von Bonsdorff CH. Brummer-Korvenkontio M, Vaheri A, Tsai TF, Regnery HL, Goldgaber D, Lee PW : Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 227 : 1041-1044, 1985
46. Lee PW, Gibbs CJ, Gajdusek DC, Yanagihara R. Serotypic classification of hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. J Clin

Microb 22 : 940-944, 1985
47. McCormick JB, Sasso DR, Palmer EL, Kiley MP : Morphological identification of the agent of Korean haemorrhagic fever (Hantaan virus) as a member of the Bunyaviridae. Lancet 1 : 765-768, 1982
48. White JD, Shirey FG, French GR, Huggins JW, Brand OM, Lee HW : Hantaan virus, aetiologic agent of Korean haemorrhagic fever, has Bunyaviridae-like morphology. Lancet 1 : 768-771, 1982
49. Hung T, Xia SM, Zhao ZY, Song G, Liao GX, Ye WW, Chu YL, Hang CS : Morphological evidence for identifying the viruses of hemorrhagic fever with renal syndrome as candidate members of the Bunyaviridae family. Arch Virol 78 : 137-144, 1983
50. Martin ML, Regnery HL, Sasso DR, McCormick JB, Palmer EL : Distinction between bunyaviridae genera by surface structure and comparison with Hantaan virus using negative stain electronmicroscoy. Arch Virol 86 : 17-28, 1985
51. Karabatsos N (ed) : International catalogue of arboviruses including certain other viruses of vertebrates, Hantaan virus. Am Soc Trop Med Hyg San Antonio Texas USA pp 445-446, 1985
52. Karabatsos N (ed) : International catalogue of arboviruses including certain other viruses of vertebrates, Seoul virus. Am Soc Trop Med Hyg San Antonio Texas USA pp 927-928, 1985
53. Karabatsos N (ed) : International catalogue of arboviruses including certain other viruses of vertebrates, Puumala virus. Am Soc Trop Med Hyg San Antonio Texas USA pp 845-846, 1985
54. Karabatsos N (ed) : International catalogue of arboviruses including certain other viruses of vertebrates, Prospect Hill virus. Am Soc Trop Med Hyg San Antonio Texas USA pp 833-834, 1985
55. Chun CH, Won OS : The analysis of death causes of 50 fatal cases in Korean hemorrhagic fever. Kor J Infect Dis

5 : 57－62, 1973
56. Chun CH, Kim SW, Kim WD, Yoon HJ : Changing pattern of epidemiology and death-causes of Epidemic hemorrhagic fever. Kor J Infect Dis 8 : 58－74, 1976
57. Chun CH : Epidemic hemorrhagic fever. Med J ROK Army 2 : 17－26, 1962
58. Chun CH : Present status of Epidemic hemorrhagic fever in Korea. Kor J Infect Dis 6 : 19－26, 1974
59. Kim WD, Chun CH, Kim MY : Korean hemorrhagic fever (KHF). Characteristics of Korean hemorrhagic fever. CRC Handbook series in Zoonoses James H Steele (Editor in chief) Section B Viral Zoonoses Vol Ⅱ CRC press Inc Boca Raton Florida 1981
60. Ishii S. Ando K, Watanabe N, Murakami R, Nagayama T, Ishikawa T : Studies on so-called Song-go fever. Med J Jap Army 355 : 1755－1758, 1942
61. Lukes RS : The pathology of 39 fatal cases of Epidemic hemorrhagic fever. Am J Med 16 : 639－643, 1954
62. Rhim JS, Chun CH : Hemorrhagic fever. Med J ROK Army 7 : 9－16, 1960
63. Lee HW, Park SB, Chu YK : Seroepidemiological study of hemorrhagic fever with renal syndrome from 1980 to 1987 in Korea. J Kor Soc Virol 2 : 131－142, 1988
64. Myhrman G : En njurejukdom med egenartad symptombild (A renal disease with a peculiar symptomatology). Nord Med Tidsskr 7 : 793－794, 1934
65. Zetterholm SG : Akuta-nefriter simulerande akuta bukfall (Cases of acute nephritis simulating acute abdomen). Lakartidn 31 : 425－429, 1934
66. Myhrman G : En ny infektionssjukdom i Nordsverige och Nordfinland (A new infectious disease in Northern Sweden and Northern Finland). Nord Med 28 : 2571－2572, 1945
67. Laehdevirta J : Clinical features of HFRS in Scandinavia as compared with East Asia. Scand J Infect Dis Suppl 36 : 93－95, 1982
68. Lee HW, Lee PW, Lahdevirta J, Brummer-Korvenkontio M : Aetiological relation between Korean haemorrhagic

fever and Nephropathia epidemica. Lancet 1 : 186-187, 1979
69. Lee JS, Cho BY, Lee MC, Choi SJ, Kim KW, Lee MH, Lee HW : Clinical features of serologically proven Korean hemorrhagic fever patients. Seoul J Med 21 : 163-169, 1980
70. Lee HW : Korean hemorrhagic fever. Prog Med Virol 28 : 96-113, 1982
71. Song G, Hang CS, Liao HX, Fu JL. Gao GZ, Qin H, Zhang QF : Antigenic comparison of virus strain of the wild-mouse type and the house-rat type of epidemic hemorrhgic fever (EHF) in China. Chin J Microb Immunol 4 : 236-238, 1984
72. Lee HW, Baek LJ, Kawamata J, Yamanouchi T : Laboratory infection of hemorrhagic fever with renal syndrome and transmission of Hantaan and related virus in rats. Abstract from the eleventh Int Cong Trop Med Mal Calgary Canada Sept 16-22, 1984
73. Kawamata J, Yamanouchi T, Dohmae K, Miyamoto H, Takahashi M, Yamanishi K, Kurata T, Lee HW : Control of laboratory acquired HFRS in Japan. Lab Anim Sci 37 : 431-436, 1987
74. Wong TW, Cheong CY, Lee HW : Hemorrhagic fever with renal syndrome in Singapore : a case report. SE Asian J Trop Med Pub Hlth 16 : 525-527, 1985
75. Morimoto Y, Kishimoto S, Yamanouchi T, Yamanishi K. Takahashi M, Kawamata J, Tamura T, Lee HW : Clinical features of hemorrhagic fever with renal syndrome in Japan : a clinical and laboratory study on 27 cases in Osaka in the 1960's and the 1980's. Jap J Infect Dis 59 : 439-458, 1985
76. Byun BS, Seo JB, Lee MS, Kim MH, Kang KH, Kim JS, Park SC, Lee HW : A clinical study of hemorrhagic fever with renal syndrome caused by Seoul virus infection. Kor J Infect Dis 18 : 11-18, 1986
77. Leung NK, Chang WK, Lee SH, Lee HW : Hemorrhagic fever with renal syndrome in Hong Kong. WHO JE HFRS Bull 2 : 51-53, 1987
78. Chan YC, Wong TW, Yap EH, Tan HC, Lee HW, Chu YK,

Lee PW : Haemorrhagic fever with renal syndrome involving the liver. Med J Austral 147 : 248-249, 1987
79. Seo TJ, Lee HW : Hemorrhagic with renal syndrome patients among suspect NA-NB hepatitis patients in Korea. Abstract 12th Pacif Sci Cong Seoul pp 225, 1987
80. Counts EF, Seltzer R : The early diagnosis of endemic hemorrhagic fever. In "Symposium on endemic hemorrhagic fever" McNinch JH editor Ann Intern Med 38 : 67-72, 1953
81. Cohen MS, Casals J, Hsiung GD, Kwei HE, Chin CC, GE HC, Hsiang CM, Lee PW, Gibbs CJ, Gajdusek DC : Epidemic hemorrhagic fever in Hubei province, the People's Republic of china : a clinical and serological study. Yale J Biol Med 54 : 41-55, 1981
82. Lee HW : Hemorrhagic fever with renal syndrome. History of Hantaan virus and epidemiological features. Scand J Infect Dis Suppl 36 : 82-85, 1982
83. Yanagihara R, Gajdusek DC : Hemorrhagic fever with renal syndrome : Global epidemiology and ecology of hantavirus infections. In : De la Maza LM, Peterson EM (Eds) : Med Virol 6 : 171 214, 1987
84. LeDuc JW : Epidemiology of Hantaan and related viruses. Lab Anim Sci 413-418, 1987
85. Lee HW : Hantavirus infection in Asia. In : Davison A.M. (Eds). Nephrology 2 : 816-831, 1988
86. Lee HW : Global update on distribution of haemorrhagic fever with renal syndrome and hantaviruses. Virus Info Exch News 5 ; No 3 82-84, 1988
87. LeDuc JW, Smith AA, Childs JE et al : Global survey of antibody to Hantaan related viruses among peridomestic rodents. Bull WHO 64 : 139-144, 1986
88. van der Groen G, Lee HW : Hemorrhagic fever with renal syndrome. Prog Med Virol In press
89. WHO, Viral hemorrhagic fevers, Report of a WHO Expert Committee, Technical Report Series 721, WHO, Geneva, 72-73, 1985
90. Lee HW, Lee PW, Baek LJ, Song CK, Seong IW :

Intraspecific transmission of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, in the rodent Apodemus agrarius. Am J Trop Med Hyg 30 : 1106 1112, 1981

91. Lee HW, Baek LJ, Kim HD : Studies on laboratory rat in fection with hantavirus in animal rooms of institutes in Seoul. J Kor Soc Virol 16 : 113 120, 1986
92. Kitamura T, Morita C, Komatsu T et al : Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. Jap J Med Sci Biol 36 : 17 25, 1983
93. Yamanishi K, Dantas JR, Takahashi M et al : Isolation of hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. Jap J. Med Sic 26 : 155 160, 1983
94. Lloyd G, Jones N : Infection of laboratory workers with Hantaan virus acquired from immunocytomas propagated in laboratory rats. J Infect Dis 12 : 2117 2125, 1986
95. Yanagihara R, Amyx HL, Gajdusek DC : Experimental infection with Puumala virus, the etiologic agent of Nephropathia epidemica, in bank voles (Clethrionomys glareolus). J Virol 55 : 34 - 38, 1985
96. Lee HW, Baek LJ, Kawamata J : Laboratory infection of hemorrhagic fever with renal syndrome and transmission of Hantaan and Seoul virus in rats. Abst 11th Int Cong Trop Med Mal Calgary Canada 84 : 16 - 22, 1984
97. Tkachenko EA, Ivanov AP, Donets MA et al : Potential reservoir and vectors of hemorrhagic fever with renal syndrome (HFRS) in the U.S.S.R. Ann Soc Belg Med Trop 63 : 267 - 269, 1983
98. Gavrilovskaya IN, Apekina NS, Miasnikov YA et al : Features of circulation of HFRS virus among small mammals in the European USSR. Arch Virol 75 : 313 316, 1983
99. Tkachenko EA, Ryltseva EV, Myasuikov A et al : Study of circulation of HFRS virus among small mammals in the USSR. Probl. Virol 6 : 709 715, 1987
100. van der Groen G, Leizs H, Verhagen R : Polyhostal nature

of hantaviruses. Proc 2nd Symp Rodent Control Kuweit Feb 6, 1985
101. Lee HW, Baek LJ, Chu YK : The study on clinical diagnosis and specific serological diagnosis of hemorrhagic fever with renal syndrome. J Kor Soc Virol 17 : 39 44, 1987
102. Lee HW : Seroepidemiologic studis of acute hemorrhagic diseases in Korea from 1985 to 1987 (Hemorrhagic fever with renal syndrome, leptospirosis and scrub typhus). J Kor Med Assoc 31 : 581 593, 1988
103. Hung T, Xia SM. Song G, Liao HX, Chao TX, Chhou ZY, Hang CS : Viruses of classical and mild forms of Hemorrhagic fever with renal syndrome isolated in China have similar Bunyavirus-like morphology. Lancet 11 : 589 591, 1983
104. Palmer EL, Martin ML : An atlas of mammalian viruses CRS Press, pp 9 11, 1982
105. Hung T, Xia SM. Chou ZY, Song G. Yanagihara R : Morphology and morphogenesis of viruses of Hemorrhagic fever with renal syndrome (HFRS) Ⅱ. Inclusion bodies-the ultrastructural markers of Hantavirus-infected cells. Intervirol 27 : 45--52, 1987
106. Kurata T. Tsai TF, Bauer SP, McCormick JB : Immunofluorescence studies of disseminated Hantaan virus infection of suckling mice. Infect Immun 41 : 391-398, 1983
107. Voller A, Bidwell D, Barlett A : Enzyme-linked immunosorbent assay, pp 359-371. In NR Rose and H Friedman (eds.) Manual of clinical immunology 2nd ed. Am Soc Microb Washington DC 1980
108. Meurman, O : Detection of antiviral IgM antibodies and its problems a review. Curr Top Microb Immunol 104 : 101-127, 1983
109. Nakane P, Kawoi A : Peroxidase-labelled antibody : a new method of conjugation. J Histochem Cytochem 22 : 1084-1091, 1974
110. Mathiesen L, Feinstone S, Wong D, Skinhoej P, Purcell R :

Enzyme-linked immunosorbent assay for detection of hepatitis A antigen in stool and antibody to hepatitis A antigen in sera : comparison with solid phase radio-immunoassay, immune electron microscopy, and immune adherence hemagglutination assay. J Clin Microb 7 : 184-193, 1978

111. Hunter W, Greenwood F : Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature 194 : 495-496, 1962
112. Purcell R, Wong D, Moritsugu Y, Dienstag J, Routenberg J, Boggs J : A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. J Immunol 116 : 349-356, 1976
113. Itoh M, Tagaya I : Immune adherence hemagglutination test as a new sensitive method for titration of animal virus antigens and antibodies. Jap J Med Sci Biol 19 : 109-126, 1966
114. Mayumi M, Okochi K, Nishioka K : Detection of Australia antigen by means of immune adherence hemagglutination test. vox Sang 20 : 178-181, 1971
115. Moritsugu Y, Dienstag JL, Valdesuso J, Wong DC, Wagner J, Routenberg JA, Purcell RH : Purification of hepatitis A antigen from feces and detection of antigen and antibody by immune adherence hemagglutination. Infect Immun 13 : 898-908, 1976
116. Matsuno S, Nagayoshi S : Quatitative estimation of infantile gastroenteritis virus antigens in stools by immune adherence hemagglutination test. J Clin Microb 7 : 310-311, 1978
117. Suzuki T, Yoshida Y, Oda K, Takamiya A : Application of immune adherence hemagglutination test for typing of enteroviruses. Virus 29 : 153-154, 1979
118. Miller WJ, Provost PJ, MacAleer WJ, Ittensohn OL, Villarejos VM, Hilleman MR : Specific immune adherence assay for human hepatitis A antibody. Application to diagnostic and epidemiological investigations. Proc Soc Exp Biol Med 149 : 254-261, 1975

119. Krugman S, Friendman H, Lattimer C : Viral hepatitis type A. Identification by specific complement fixation and immune adherence tests. N Engl J Med 292 : 1141-1143, 1975
120. Gershon AA, Kalter ZG, Steinberg S : Detection of antibody to varicella-zoster virus by immune adherence hemagglutination. Proc Soc Exp Biol Med 151 : 762-765, 1976
121. Lennette ET, Lennette DA : Immune adherence hemagglutination : alternating to complement-fixation serology. J Clin Microb 7 : 282-285, 1978
122. Inouye S, Matsuno S, Kono R : Difference in antibody reactivity between complement fixation and immune adherence hemagglutination tests with virus antigens. J Clin Microb 14 : 241-246, 1981
123. Sugiyama K, Matsuura Y, Morita C, Shiga S, Akao Y, Komatsu T, Kitamura T : An immune adherence assay for discrimination between etiologic agents of hemorrhagic fever with renal syndrome. J Infect Dis 149 : 67-73, 1984
124. Sugiyama K, Matsuura Y, Morita C, Morikawa S, Komatsu T, Shiga S, Akao Y, Kitamura T : Determination by immune adherence HA of the antigenic relationship between Rattus and Apodemus-borne viruses causing hemorrhagic fever with renal syndrome. J Infect Dis 149 : 472-473, 1984
125. Tsai TF, Tang YW, Ye KL, Chen GL, Xu ZY : Hemagglutination inhibition antibody in hemorrhagic fever with renal syndrome. J Infect Dis 150 : 895-898, 1984.
126. Brummer-Korvenkontio M, Manni T, Ukkonen S, Vaheri A : Detection of hemagglutination-inhibiting antibodies in patients with Nephropathia epidemia and Korean hemorrhgic fever by using Puumula virus cell culture antigen. J Infect Dis 103 : 997-998, 1986
127. Okuno Y, Yamanishi K, Takahashi Y et al : Haemagglutination-inhibition test for hemorrhagic fever with renal syndrome using virus antigen prepared from infected tissue culture fluid. J Gen Virol 67 : 149-156, 1986

128. Clarke DH, Casals J : Techniques for hemagglutination and hemagglutination inhibition with arthropod borne viruses. Am J Trop Med Hyg 7 : 561 573, 1958
129. Shope RE, Sather GE : In : Lennette EH, Schmidt NJ eds., Diagnostic procedures for viral and rickettsial infections. Washington DC : Am Pub Hlth Assoc 767 814, 1980
130. French GR, Foulke RS, Brand OA, Eddy GA, Lee HW, Lee PW : Korean hemorrhagic fever : propagation of the etiologic agent in a cell line of human origin. Science 211 : 1046 1048, 1981
131. Catalogue of Strains II : American Type Culture Collection, Rockville, MD. USA, 4th Edition, 145 146, 1983